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FOLIC ACID

BY

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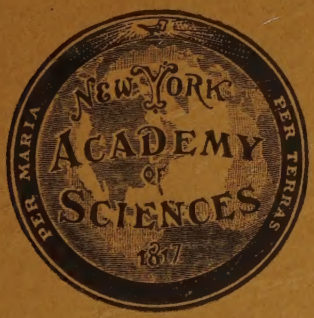

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FOLIC ACID*

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HISTORY OF THE FOLIC ACID FACTORS

By W. H. Peterson

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

The discovery of the several compounds called collectively, for want of a suitable chemical name, "folic acid," is the result of many lines of work. One central purpose has actuated all of these efforts, namely, the determination of the nutritional requirements of animals and bacteria. The investigations in widely separated fields have converged, until they have met in the isolation, characterization, and finally the synthesis, of the vitamin. Although I am not a bacteriologist by profession, it gives me particular pleasure to note the outstanding contribution that has come from the use of bacteria in this successful effort. The bacteriologists are perhaps the oldest experimental nutritionists. For them, it has been a practical day-by-day problem, from the time when they began to carry pure cultures. They early realized the importance of extracts of beef, blood, milk, yeast, and other biological materials, though generally without attempting to learn the nature of the constituents that were responsible for the good effects of these biological materials. It has long been my belief that, if vitamin research had from the beginning been conducted simultaneously with animals and with bacteria, we would have attained our present knowledge of vitamins a decade or more earlier.

The multiplicity of names applied to preparations possessing folic acid potency came about because of independent and unconnected investigations dealing with deficiencies encountered in studying the nutrition of animals and bacteria. Names that preceded "folic acid" are "vitamin M," applied to a factor needed for the monkey, by Day, Langston, and Darby[1]; "factor U", by Stokstad and Manning,[2] "factors R and S," by Schumacher, Heuser, and Norris,[3] and "vitamin B_c," by Hogan and Parrott,[4] all used to designate substances required by the chick; and "norite eluate factor," required by *Lactobacillus casei*, by Snell and Peterson.[5, 6] In 1941, the term "folic acid" was applied by Mitchell, Snell, and Williams[7] to a factor required by *Streptococcus lactis* R (later shown to be a strain of *Streptococcus faecalis*), but also potent for *L. casei*. Later, the same authors[8] limited the name to the factor required by *S. lactis* R. Parenthetically, it may be of some slight interest to explain that the letter, R, used for many years to designate this strain in the laboratories of the Univer-

sity of Wisconsin, was chosen because the culture was originally obtained from Dr. Lore A. Rogers, Bureau of Dairy Industry, U.S. Department of Agriculture, Washington. For a score of years, this microorganism remained in the literature in comparative obscurity, when it was suddenly brought into prominence because of its use as a test agent for members of the folic acid group. Following the introduction of the name, *folic acid,* other terms such as "vitamins B_{10} and B_{11}" for the chick (Briggs, Luckey, Elvehjem, and Hart[9]), "guinea pig factor 1" (Woolley and Sprince[10]), "*L. casei* factor," potent especially for *L. casei* (Hutchings, Stokstad, Bohonos, and Slobodkin[11]), and "factor SLR" for *S. lactis* R (Keresztesy, Rickes, and Stokes[12]), have all been applied to substances having folic acid potency. This list is not complete, for there have been many other papers, both in this country and in England, that have dealt with deficient rations and deficient bacteriological media that can be improved by the addition of folic acid.

If one were to point to lines of work that have been outstanding in the detection and isolation of the elusive and many-sided vitamin, it would be to investigations involving the chick and bacteria. I well remember that, in the early thirties, the use of the chick as a test animal was looked upon with misgivings by many investigators in the field of nutrition. Even fewer workers in the animal field accepted bacteria as an aid to vitamin study. That is all changed today, for, in the unraveling of the B-complex, microbiology has played just as important a part as animal work. According to my score card, 4 of the 10 vitamins in the B-complex should be credited to microbiology, 4 to animal work, and two, one of which is folic acid, should be assigned to both fields. This interpretation does not imply that workers in microbiology and animal research are antagonists. They should be, and usually are, friendly though keen rivals.

I presume that I have followed that digression far enough, and will return to the story of folic acid. In 1941, it appeared to the men working on chick vitamins and bacterial growth-factors in our laboratory that we were probably dealing with the same factor. At that time, we had succeeded in concentrating the bacterial factor from solubilized liver about 200-fold, and when this preparation was fed to chicks it was found to have the same comparative potency for the chick as for *L. casei.* Likewise, loss of potency on storage was parallel for both bacteria and chick. A letter[13] to the Editor of the Journal of Biological Chemistry on the parallelism between the chick and bacterial factors, and later a more extensive paper[14] on the concentration and

properties of the factor, established a bridge between the two fields of investigation which has continued not only in our laboratory, but also in pharmaceutical laboratories where the several forms of the vitamins have been isolated. For several years, the method described in that paper for the concentration of the factor was the only one in print and was used by a number of laboratories. In 1944, Mitchell, Snell, and Williams[15] published a detailed procedure for concentrating folic acid from spinach to a very high degree: 137,000 times that in the liver product (B) which they used as a standard. Rather anomalously, the work which gave rise to the name of *folic acid* did not yield a crystalline compound. In the preceding year, crystalline forms of the vitamin had been reported by Pfiffner *et al.*,[16] Keresztesy *et al.*,[12] and by Stokstad.[17] Also in 1944, a fourth report (Hutchings *et al.*[11]) was published, stating that a crystalline compound had been obtained. None of these reports gave information as to how the crystalline compounds were obtained, and some did not reveal the source material from which the compound was isolated. Happily, the curtain which for so long has hidden many aspects of this work is to be lifted here.

The detection and isolation of folic acid have been complicated by the many forms this vitamin can assume. To date, as I have already indicated, four different crystalline compounds have been obtained from liver, yeast, and other natural sources. These have been designated as "vitamin B_c," "Factor SLR," and "*L. casei* factor." Three forms of the *L. casei* factor have been obtained from liver, yeast, and a fermented medium, respectively. The compound from liver appears to be identical with vitamin B_c. A fifth compound that appears to be an entity is the conjugated form of vitamin B_c.[18] These several compounds have different chemical and biological properties, which largely explains the confusion that has attended the identification of the vitamin. In recent months, a good deal of this confusion has been cleared up by treating the several compounds with enzymes and testing the products thus obtained, simultaneously by means of bacteria, the chick, and the monkey. The unity that is emerging in the picture should be still more apparent now that the chemistry of the *L. casei* factor is known. Announcement of the chemistry of this factor is the most important purpose of this conference. It is to be hoped that, as a result, a suitable chemical name can be given to the vitamin.

In closing, I wish to say a word of appreciation to the committee which arranged this conference, and to Dr. SubbaRow in particular, for inviting me to participate. I consider myself merely the representative of many colleagues and assistants who have all contributed to the work

at our institution. We take particular pride in the fact that some of the students who started their work in our laboratories have carried the research to other places and have had such a conspicuous part in its development. I would like to name two, especially: Esmond E. Snell, who continued the work so successfully at the University of Texas, but who is now back with us as a permanent member of the faculty; and Brian L. Hutchings, who went to the Lederle Laboratories in 1941 and who, because of his notable work there, has contributed to this publication.

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ISOLATION OF THE LIVER *L. CASEI* FACTOR

By E. L. R. Stokstad, Brian L. Hutchings, and Y. SubbaRow

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In the isolation of the liver *Lactobacillus casei* factor from liver, four essential steps were used. These were: adsorption and elution, esterification and extraction of the methyl ester with immiscible solvents, chromatographic adsorption of the ester, and fractional precipitation of the ester from water and methanol. The activity of the various fractions was followed by microbiological assay, using *L. casei*. The assay results were expressed in terms of an arbitrary unit which is the amount required per 10 ml. of medium for three-fourths maximum growth. The pure liver *L. casei* factor was found to have an activity of approximately 1,000,000 units per mg.

The starting material for this isolation was a dried 85% ethanol precipitate of an aqueous extract of liver. This preparation, which will be referred to in the discussion as liver extract, contained 20,000 units of activity per gram. This liver extract was first dissolved in water, brought to pH 8.5, heated to 80° C., and $CaCl_2$ was added to flocculate the precipitate which formed. The *L. casei* factor was adsorbed from this filtrate by treatment with norite at pH 3.0. This adsorbate was washed first with neutral 60 per cent ethanol to remove inert materials, and then with 0.5 N NH_4OH in 60 per cent ethanol at 70° C. to remove the activity. This eluate contained 7,000 units per gram equivalent of original liver extract.

The eluate was concentrated to 1 liter per Kg. of original material and adjusted to pH 3.5. A large amount of inert material precipitated out, but approximately half of the activity was carried down with it. The pH 3.5 filtrate contained 3,500 units per gram of original liver extract. This filtrate was adsorbed at pH 1.3 on superfiltrol by percolation through a column of the granular adsorbent. Elution was effected by percolation with 0.5 N NH_4OH in 60 per cent ethanol. After elution, the adsorbent was washed with dilute acid, and re-used for another adsorption and elution. When an adsorbent is used only once, a certain amount of activity is adsorbed which cannot be eluted. In subsequent adsorptions and elutions, this loss does not occur. After 6 adsorptions and elutions, no decrease in efficiency of the adsorbent was observed, and almost complete recovery of activity was obtained.

The superfiltrol eluate contained 3,500 units per gram equivalent of liver extract and 340 units per mg. of solids. This represents a 17-fold increase in activity and a 17 per cent recovery.

The superfiltrol eluate was concentrated and neutralized to pH 7.0. The *L. casei* factor was then precipitated as the barium salt by adding ethanol to a concentration of 90 per cent and adding an excess of $BaCl_2$. Though little or no increase in activity was achieved by this step, it converted the material into a form which could be dried, finely ground, and then esterified. It should be noted that, while the *L. casei* factor can be precipitated by heavy metals such as lead, silver, and by basic precipitants such as phosphotungstic acid, no large increases in activity could be effected at this stage by the use of these reagents.

The next step consisted in esterification and extraction of the methyl ester with *n*-butanol. Esterification was carried out by dissolving the barium salt in 0.2 N HCl methanol. The reaction proceeded rapidly, coming to completion in 1 hour at 25° C. The ester was approximately 10 per cent as active, microbiologically, as the free acid. Activity assays were preceded by 10 minutes saponification with 0.1 N NaOH at 100° C. The esterified mixture was neutralized and evaporated to dryness, redissolved in water, and adjusted to pH 6 to 7. It was then extracted 3 times with 2 volumes of *n*-butanol. Each butanol extract was washed successively with the same portion of a half-volume of water. The distribution coefficient of the *L. casei* factor methyl ester for butanol to water is 3 to 1. The final butanol extract contained 2,000 units per gram equivalent of liver and 3,450 units per mg. of solids.

While chromatographic adsorption of the free acid from aqueous solution proved ineffective, chromatographic adsorption of the ester in organic solvents was highly efficient. Superfiltrol proved the best adsorbent; Brockman's alumina and $CaCO_3$ were much less effective. The *L. casei* factor methyl ester could be adsorbed from butanol, methanol, acetone, and water. The only efficient eluant was aqueous acetone. Mixtures of various alcohols and aqueous alcohol solutions were ineffective. By eluting first with 92.5 per cent acetone, it was possible to remove considerable impurities without losing much activity. Elution with 75 per cent acetone rapidly removed the active material. The 75 per cent acetone eluate was divided into several fractions. The first of these contained 1,200 units per gram equivalent of liver and 47,000 units per mg. of solids.

The next increase in activity was achieved by fractional precipitation of the ester from water and methanol. The 75 per cent acetone

eluate was evaporated until most of the acetone had been removed. On cooling the resulting aqueous solution, most of the activity precipitated out. This contained 1,000 units per gram of original liver and 370,000 units per mg. of solids. This "water precipitate" was extracted with a small amount of cold methanol, 1 ml. of methanol being used per Kg. of original liver. This removed most of the dark brown pigment. The *L. casei* factor methyl ester could be dissolved by extraction with a larger amount of hot methanol (4 ml. per Kg. original liver). This hot methanol extract was almost colorless and highly active; it contained 780 units per gram of original liver and 950,000 units per mg. of solids. This represents almost pure material.

On cooling the hot methanol extract, the methyl ester separated out in nearly pure form as a gelatinous precipitate. Reprecipitation of this methanol yielded a preparation whose activity could not be increased by further purification.

Two preparations of this were analyzed as follows:

	C	H	N
Sample 1	53.0	4.6	21.2
Sample 2	53.1	5.1	20.5

The activity of the pure ester (after saponification) was 1,000,000 units per mg.

The free acid was prepared by saponifying a nearly pure preparation of the ester with 0.1 N NaOH at 25° C., and treating the alkaline solution with a small quantity of activated charcoal. On acidification to pH 3.0, the free acid precipitated out. It was crystallized by dissolving in hot water and allowing to cool.

The extinction coefficients of the free acid in 0.1 N NaOH were as follows:

mμ	$E^{1\%}_{1\text{ cm.}}$
256	565
283	550
365	195

ISOLATION OF THE FERMENTATION *L. CASEI* FACTOR

By Brian L. Hutchings, E. L. R. Stokstad, Nestor Bohonos, Nathan Sloane, and Y. SubbaRow

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The source material for the isolation of the fermentation *Lactobacillus casei* factor was a filtrate obtained from an aerobic fermentation of an unidentified bacterium of the genus *Corynebacterium*. The liquor contained from 3 to 5 micrograms of the active compound per ml.

The activity of the compound was followed by microbiological assay with *Lactobacillus casei*, according to established methods.

The crystalline compound was obtained by the following procedure. After removal of the bacterial cells, the solution was adjusted to pH 3.0, and 6 grams of Norite A were added per liter of filtrate. After 30 minutes adsorption, the charcoal was filtered off and washed well with water. The charcoal was eluted with 50 per cent ethanol (6 liters per Kg. of charcoal). This eluate was discarded. The charcoal was then eluted with 50 per cent ethanol and 10 per cent ammonium hydroxide (by volume) at 70° C. for 1 hour (12 liters per Kg. of charcoal). The elution was repeated once. The eluates were combined. The recovery approximated 65 per cent.

The ammonia-ethanol eluates were adjusted to pH 8.0, with concentrated hydrochloric acid and ethanol added to a concentration of 85 per cent. A saturated aqueous solution of barium chloride was added until complete precipitation occurred, meanwhile maintaining the pH at 8.0. After cooling to 0°–5° C., the precipitate was centrifuged out and washed with methanol. The active compound was completely precipitated by this procedure.

The barium precipitates were suspended in 0.25 N methanol-hydrogen chloride (1/15th the volume of the original filtrate) and esterified at room temperature. At the end of 24 hours, the esterification mixture was adjusted to pH 4.5 by cautious addition of 5 N sodium hydroxide, and then concentrated to dryness under reduced pressure. The residue was suspended in a volume of water equivalent to a concentration of 15 micrograms per ml. of the active compound. The water insolubles were centrifuged out and discarded.

The aqueous solution was extracted 3 times with 2 volumes of bu-

tanol. Each butanol extract was in turn extracted with ½ volume of water. The combined butanol extracts were concentrated to ⅙ of their volume and extracted with ½ volume of water. This water wash was extracted with 2 volumes of butanol. The butanol extracts were combined and concentrated to dryness under reduced pressure. The esterification and butanol extraction gave a yield of 50–90 per cent.

The residue after removal of the butanol was dissolved in the minimum amount of hot methanol. After thorough chilling to −5° C., the precipitated ester was collected. The precipitate was extracted 2 times (total volume was ⅕ the volume of methanol necessary to dissolve the residue after removal of the butanol) with 0.1 N methanol-hydrogen chloride. The extracts were diluted with 2 volumes of methanol, and 2 moles of sodium acetate were added per mole of hydrogen chloride. The solution was heated to 60° C., centrifuged, and the supernatant chilled at −10° C. for 24 hours. The precipitate which formed was centrifuged, then dissolved in hot methanol, and sodium chloride was added to a concentration of 0.05 N. The solution was centrifuged at 60° C., and the insoluble fraction discarded. The supernatant was cooled at −10° C. for 24 hours, and then centrifuged at 2° C. The precipitated ester was obtained in yields of around 65 per cent.

The ester was washed free of methanol, suspended in water, and 0.1 N barium hydroxide was added until the solution was faintly alkaline to phenolphthalein. The hydrolysis of the ester was extremely rapid. The hydrolysate was centrifuged and the supernatant treated with florisil (1 gram per 100 mgs. of active compound) for ½ hour, to remove extraneous pigments. The florisil was filtered off and washed with dilute barium hydroxide. The filtrate and washings were combined. The yield was 85–90 per cent.

One-tenth of a volume of 1.0 N barium chloride was added, the solution was cooled to 0°–5° C., and ethanol was added to a concentration of 50 per cent. The solution was chilled overnight in the refrigerator, then centrifuged, and the precipitate washed with alcohol and ether, and dried. All of the active compound was found in the precipitate.

The barium precipitate was extracted with hot water. The resulting extracts were combined, and 1 N hydrochloric acid was added to pH 2.8. The solution was cooled to 0°–5° C., and the precipitate collected. The precipitate was dissolved in hot water previously adjusted to pH 2.8 and containing a small amount of calcium or sodium chloride. On cooling, the acid precipitated as very short needles or

long threads. The compound could be repeatedly crystallized in this manner.

The analyses of the compound were:

Sample No.	C	H	N
1	48.6	4.8	16.2
2	48.0	4.4	15.5
3	47.7	4.7	15.5

To date, difficulty has been experienced in obtaining consistent analyses.

The fermentation *Lactobacillus casei* factor exhibits the same absorption characteristics as the liver *L. casei* factor.[1] The extinction coefficients are somewhat lower, indicating that the fermentation *L. casei* factor is a higher molecular weight compound. The extinction coefficient at 365 mμ in 0.1 N sodium hydroxide is 134.

The compound can be re-esterified, using 0.1 N methanol-hydrogen chloride. The ester can be crystallized as short needles or long threads from methanol 0.05 N with sodium chloride.

The fermentation compound has the same biological activity for animals as does the liver *L. casei* factor, with the proviso that increased amounts are necessary to compensate for the higher molecular weight.

The fermentation *L. casei* factor is distinguished from the liver *L. casei* factor by its relative activity for *Lactobacillus casei* and *Streptococcus faecalis* R. The fermentation compound is 60–80 per cent as active as the liver compound for *L. casei*, but only 6 per cent as active for *S. faecalis* R. The amounts required per ml. for half-maximum growth of *L. casei* and *S. faecalis* R., respectively, are 0.000061 micrograms and 0.0042 micrograms.

The analyses and biological activity serve to distinguish this compound from any of the compounds previously reported as influencing the growth of *L. casei* or *S. faecalis* R under conditions of the test.

SUMMARY

An isolation procedure for the fermentation *L. casei* factor is outlined. The analyses and biological activity distinguish the fermentation *L. casei* factor from any similar compound previously reported.

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DEGRADATION OF THE FERMENTATION *L. CASEI* FACTOR

I

By E. L. R. Stokstad, Brian L. Hutchings, John H. Mowat, James H. Boothe, Coy W. Waller, Robert B. Angier, Joseph Semb, and Y. SubbaRow

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In experiments on the degradation of the *Lactobacillus casei* factors, it was observed that hydrolysis with acid or alkali led to the formation of a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall.[1] The liberation of the amine was most rapid in alkaline solutions, and it was soon found that oxygen had a marked effect on the course of the reaction.

When the fermentation *L. casei* factor was heated with 1.0 N NaOH at 100° C. in the presence of oxygen, there was a marked change in absorption spectra, an aromatic amine was formed, a fluorescent pigment was produced, and rapid biological inactivation occurred. However, when this fermentation compound was hydrolyzed anaerobically for 10 hours at 120° C., no aromatic amine or fluorescent pigment was produced, and no change in absorption spectrum took place. The activity for *L. casei* was only slightly decreased during anaerobic hydrolysis, while the activity for *S. faecalis* R was greatly increased. At the same time, 25 per cent of the nitrogen appeared as alpha amino acid nitrogen. This alpha amino acid nitrogen had been cleaved from the rest of the molecule, because it could be separated from the biologically active fragment. This alpha amino acid nitrogen was probably present as a dicarboxylic alpha amino acid, because it could be precipitated by $Ba(OH)_2$ and 75 per cent ethanol.

Anaerobic hydrolysis with 1.0 N NaOH for 10 hours at 120° C. increased the activity for *S. faecalis* R, until the ratio of the activities for *L. casei* and *S. faecalis* R was nearly the same as that for the liver *L. casei* factor. The biologically active compound obtained by anaerobic hydrolysis was found to be *dl* liver *L. casei* factor. This was shown by comparison of its infra-red absorption spectrum with that of the synthetic *dl* liver *L. casei* factor. It is very probable that the *dl* form

[1] **Bratton, C. A., & E. K. Marshall.** J. Biol. Chem. **128**: 537. 1939.

was produced by racemization, during the extended alkaline hydrolysis. Thus, it appears that the fermentation *L. casei* factor can be split into the liver *L. casei* factor plus two moles of a dicarboxy alpha amino acid.

The fluorescent pigment which was produced by 4 hours of aerobic hydrolysis with 1.0 N NaOH at 100° C. was isolated by acidifying the hydrolysate to pH 3.0. It was crystallized as the sodium salt from 2.0 N NaOH, but could not be crystallized from weakly alkaline solutions. The free acid prepared by precipitation at pH 3.0 was amorphous, and all attempts to produce a crystalline product were unsuccessful. However, this amorphous free acid did show a microcrystalline structure by X-ray diffraction.

Elementary analysis suggested the empirical formula $C_7H_5N_5O_3$.

	C	H	N
Found	40.1	1.75	32.2
Theory	40.6	2.42	33.8

It should be noted that reproducible analyses, especially on nitrogen, were difficult to obtain with these compounds.

A titration curve of the sodium salt showed it to be a dibasic acid with one pKa at 3.9 and another at 7.7. The equivalent weight of the sodium salt was 145.

The presence of a carboxylic acid group was demonstrated by decarboxylation at 300° C. for 3 hours. This liberated 0.8 mole of CO_2. From this decarboxylated product, a new fluorescent monobasic substance was obtained with a pKa of 8.0. This decarboxylation of a dibasic acid to a monobasic acid with a pKa of 8.0 shows that the original compound contained a carboxylic and an enolic group. This decarboxylated fraction had ultraviolet absorption spectra in 0.1 N NaOH with maxima at 253 and 365 mμ, while the original dibasic acid had maxima at 262 and 365 mμ.

The presence of a substituted guanidine group in the fluorescent dibasic acid was observed. Oxidation with chlorine water and subsequent hydrolysis with .1 N HCl at 140° C. for 3 hours yielded guanidine which was estimated by colorimetric methods.

Thus, the evidence indicates that the fluorescent dibasic acid contains a carboxylic group, an enolic group, and a substituted guanidine. The formation of guanidine by chlorine oxidation is evidence for a pyrimidine ring with an amino group in the 2 position. Pyrimidines, purines, and pterins which contain a 2-amino group liberate guanidine under

these conditions. The absorption spectrum of the fluorescent dibasic acid in 0.1 N NaOH is characterized by two strong maxima at 262 and 365 mμ. This is evidence for a pteridine ring, as no purines or pyrimidines have absorption maxima above 300 mμ. The fluorescence also suggests a pteridine ring. This compound was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid. The synthesis and proof of structure will be given in a later paper.

Hydrolysis with 1.0 N H_2SO_4, anaerobically for 8 hours at 100° C., yielded a fluorescent monobasic acid. This was separated by butanol extraction from any traces of the dibasic acid pterin which may have been present. This butanol extraction removed the monobasic compound and left the dibasic acid pterin in the aqueous phase. This monobasic compound was crystallized as the sodium salt from 10.0 N NaOH and was then converted to the free acid. This was identified as 2-amino-4-hydroxy-6-methylpteridine, by comparison of the ultraviolet and infra-red absorption spectra of the natural and synthetic compounds. The synthesis and proof of structure of this compound will be given in a later paper.

It should be noted that this pterin contains a methyl group in the 6 position, while the dibasic acid pterin obtained by aerobic alkaline hydrolysis contains a carboxy group in the 6 position. Evidence which will be presented in a later paper indicates that this methyl group does not exist preformed in the original *L. casei* factor.

The aromatic amine fraction was obtained by hydrolyzing the *dl* liver *L. casei* factor with 1.0 N NaOH for 4 hours at 100° C. in a stream of oxygen. The pterins were removed from this hydrolysate by precipitation with $AgNO_3$ at pH 3.0. The amine was then precipitated as the barium salt with $Ba(OH)_2$ and ethanol. This shows the amphoteric nature of the aromatic amine. The reaction of this amine to the Bratton and Marshall test indicates a substituted aromatic amine. The Bratton and Marshall test consists in diazotizing the amine with nitrous acid and then coupling with N-(l-napthyl) ethylene diamine dihydrochloride to form a red pigment. Those aromatic amines with a negative group in the *meta* or *para* position, such as *p*-aminobenzoic acid, *p*-amino acetophenone, and sulfonamides, form a color which develops rapidly, reaching a maximum in 3 minutes. *Ortho*-aminobenzoic acid and other amines which contain no negative substitution, such as aniline or toluidine, develop a color much more slowly, several hours being required to react a maximum. The rapid development of color by the aromatic amine from the *L. casei* factor suggests a *meta* or *para* substituted amine.

The aromatic amine obtained from *dl* liver *L. casei* factor contained 2.1 atoms of total nitrogen for each atom of aromatic amine nitrogen. This was based on the assumption that the aromatic amine gave the same molal color with the Bratton and Marshall test as *p*-aminobenzoic acid. The distribution coefficient of this aromatic amine, at pH 3.0 for ethyl acetate to water, was 0.2 to 1.0. That for *p*-aminobenzoic acid under the same conditions was 11 to 1.0. On hydrolysis with 2.0 N H_2SO_4 for 16 hours at 100°, 45 per cent of the total nitrogen appeared as alpha amino acid nitrogen, and the distribution coefficient of the aromatic amine became the same as that for *p*-aminobenzoic acid. *Para*-aminobenzoic acid was isolated from this hydrolysate and identified by its melting-point and by microbiological assay with *Clostridium acetobutylicum* and with *Acetobacter suboxydans*. These results show that the aromatic amine from *dl* liver *L. casei* factor is a dipeptide of *p*-aminobenzoic acid and an alpha amino acid. The linkage involves the carboxyl group of the *p*-aminobenzoic acid, as the Bratton and Marshall test reacts only with a primary aromatic amine nitrogen.

Some evidence regarding the mode of linkage is furnished by the results of aerobic alkaline hydrolysis. The absence of fluorescence and free aromatic amine in the original *L. casei* factor, and the simultaneous appearance of these two at the same rate during aerobic alkaline hydrolysis, suggest that the pterin is linked to the aromatic amine nitrogen. Biological inactivation parallels amine and pterin formation. No method of cleavage has been found which will liberate the aromatic amine without forming a pterin.

The requirement of oxygen during this cleavage demands a linkage which is stable to alkali and which can be split only by oxidative hydrolysis. This excludes an amide linkage between the 6-carboxy group of the pterin and the amine of *p*-aminobenzoic acid.

The results of these degradations can be summarized as follows:

1. Anaerobic alkaline hydrolysis of the fermentation *L. casei* factor yields *dl* liver *L. casei* factor and 2 moles of a dicarboxy alpha amino acid.

2. Aerobic alkaline hydrolysis gives 2-amino-4-hydroxypteridine-6-carboxylic acid and an aromatic amine.

3. Anaerobic acid hydrolysis yields 2-amino-4-hydroxy-6-methylpteridine.

4. The aromatic amine obtained by aerobic alkaline hydrolysis of *dl* liver *L. casei* factor consists of dipeptide of *p*-aminobenzoic acid and an alpha amino acid.

5. Linkage of the pterin to the amino group of *p*-aminobenzoic acid is indicated by the rates of liberation of pterin and amine during aerobic alkaline hydrolysis.

II

By Brian L. Hutchings, E. L. R. Stokstad, John H. Mowat, James H. Boothe, Coy W. Waller, Robert B. Angier, Joseph Semb, and Y. SubbaRow

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In the previous paper, evidence was presented indicating that the 2-amino-4-hydroxypteridine-6-carboxylic acid and *p*-aminobenzoic acid were degradation products of the fermentation *Lactobacillus casei* factor.[1] In this paper, the compounds arising from aqueous hydrolysis and sulfurous acid hydrolysis will be described.

When the fermentation *L. casei* factor was dissolved in water at pH 4.0 at a concentration of 1.6 mgs. per ml., and autoclaved at 120° C. for 15 hours, the biological activity of the compound was destroyed. On cooling the solution, a precipitate formed that was discarded. The filtrate was concentrated to dryness and extracted with absolute ethanol. The ethanol was removed under reduced pressure and the residue extracted with acetone. The acetone extracts were concentrated to dryness and sublimed at 148° C. in high vacuum for 6 hours. By this procedure, a crystalline acid was obtained.

The analysis was:

C	47.05,
H	5.14,
N	10.93.

The sublimate melted at 147°–148° C.* A cryoscopic determination of the molecular weight, using camphor as the solvent, gave a value of 284.

The compound exhibited no ultraviolet absorption. A Bratton and Marshall[2] test for aromatic amine was negative. The substance gave no color with ferric chloride in aqueous or alcoholic solution. The sample adsorbed no hydrogen over Adam's PtO_2 catalyst.

The compound contained no alpha amino acid nitrogen. However,

[1] **Stokstad, E. L. R., *et al.*** Ann. N. Y. Acad. Sci. **48**(5): 269. 1946.
[2] **Bratton, A. C., & E. K. Marshall.** J. Biol. Chem. **128**: 537. 1939.
* All melting points are uncorrected.

on hydrolysis with 2 N alkali for 3 hours at 100° C., the nitrogen was converted into alpha amino acid nitrogen.

The melting-point, analysis, and conversion of the nitrogen into alpha amino acid nitrogen indicated the compound to be pyrrolidonecarboxylic acid. The molecular weight determination suggested the possibility of an anhydride formed from 2 moles of glutamic acid.

Either compound, on hydrolysis, would yield glutamic acid. When the alkaline hydrolysate of the sublimate was assayed microbiologically, 1 mole of glutamic acid was indicated. As the pyrrolidonecarboxylic acid was the most likely compound, this was synthesized[3] and compared with the unknown. The melting-point of the pyrrolidonecarboxylic acid was 144°–148° C. The melting point of the unknown was 147°–148° C. A mixture of the unknown and pyrrolidonecarboxylic acids melted at 145°–147° C., indicating the identity of the two samples. Further corroborative evidence of the identity of the unknown with pyrrolidonecarboxylic acid was obtained by a comparison of their infra-red absorption spectra. The spectra are identical.

The compound arising from aqueous hydrolysis of the fermentation *L. casei* factor was pyrrolidonecarboxylic acid. As the degradative conditions would favor cyclization of glutamic acid, this compound was presumed to be the primary product of cleavage.

When the fermentation *L. casei* factor was dissolved in 0.5 N sulfurous acid at a concentration of 1 mg. per ml., and allowed to stand at 30° C. for 16 hours, the growth-promoting properties of the compound were destroyed. There was a marked increase in the fluorescence of the solution, and an aromatic amine was formed that could be detected by the method of Bratton and Marshall.

After removal of the sulfur dioxide, the pterin moiety reacted rapidly with aldehyde reagents, such as phenyl hydrazine, to form insoluble derivatives, thus suggesting the presence of a carbonyl group.

When the freshly prepared sulfurous acid hydrolysates were extracted with butanol at pH 3.0 and 7.0, the distribution coefficients of the fluorescent compound at the two pH values were the same, indicating the absence of a carboxylic acid group. If the compound was allowed to stand in dilute alkali anaerobically, the fluorescence of the solution increased. The distribution coefficients between butanol and water at pH 3.0 and 7.0 were indicative of the formation of a fluorescent compound containing a carboxylic acid group. From this solution, a compound crystallized out and was identified as the 2-amino-4-hydroxypteridine-6-carboxylic acid. The formation of the 6-carboxylic

[3] **Abderhalden, E., & K. Kartzsch.** Z. für Physiol. Chem. **68**: 487. 1910.

acid suggested that the aldehyde was undergoing a Cannizzaro-type dismutation, and that a neutral compound was also being formed. Accordingly, the solution was adjusted to pH 7.0 and extracted with 3–10 volume portions of butanol. The butanol extracts were concentrated to a convenient volume and the compound precipitated by the addition of ether. The precipitate was collected, dried, and then crystallized from 5 N sodium hydroxide. After several recrystallizations, the compound was converted to the free acid and dried for analysis:

			Theory for $C_7H_7N_5O$
C	44.61	47.4	47.4
H	4.17	4.43	3.95
N	37.3	39.6	39.5
Ash	5.7		

The ultraviolet absorption spectra of the compound indicated its identity with the 2-amino-4-hydroxy-6-methylpteridine. The synthesis and proof of structure of this compound will be presented in the succeeding paper. The 6-carboxylic acid and the 6-methyl compound were formed in approximately equal amounts.

From the evidence presented, it is apparent that sulfurous acid cleavage of the fermentation *L. casei* factor yields a pteridine aldehyde which dismutates in alkali to form approximately equal amounts of the 2-amino-4-hydroxypteridine-6-carboxylic acid and the 2-amino-4-hydroxy-6-methylpteridine.

The amine fragment was purified in the following manner: The pteridine fraction was removed by precipitation with silver at pH 2.0. After precipitation of excess silver ions, the amine was precipitated by the addition of a solution of lead acetate at pH 5.0. The lead precipitate was collected, suspended in water, and decomposed as the sulfide. After removal of the lead sulfide, the solution was made alkaline to phenolphthalein with barium hydroxide, and the amine precipitated by the addition of 1 volume of ethanol. The barium salt was dissolved in water, the solution adjusted to pH 2.8, and concentrated to dryness under reduced pressure. The compound was extracted into anhydrous isopropanol and precipitated by the addition of 1 volume of petroleum ether. This precipitation was repeated once. The amine was dissolved in water, the pH adjusted to 8.0 with barium hydroxide, and the compound precipitated by the addition of 1 volume of ethanol. The amine was washed thoroughly with ethanol and dried for analysis.

The free acid was readily soluble in alcohol and water, but could not be crystallized out of these solvents or combinations thereof. The free acid was extremely hygroscopic and could not be satisfactorily handled as such. The barium salt was relatively non-hygroscopic and was satisfactory for analytical purposes.

The analytical values were:

		Free acid
C	32.46	49.8
H	4.20	6.44
N	6.64	10.17
Barium	34.80	

When the amine fragment was hydrolyzed with 1 N hydrochloric acid, 75 per cent of the nitrogen was converted into alpha amino acid nitrogen. 25 per cent of the nitrogen was present as aromatic amine nitrogen. The aromatic amine was isolated by extraction with ethyl acetate, and then crystallized from water.

Analysis	Theory for *p*-aminobenzoic acid
C 61.5	61.3
H 4.93	5.11

The compound melted at 180.5°–182.5° C. An authentic sample of *p*-aminobenzoic acid melted at 183.5°–184.5° C., and a mixture of the unknown and *p*-aminobenzoic acids melted at 182.5°–183.5° C. The unknown was identical with *p*-aminobenzoic acid.

Microbiological assay of the acid hydrolysates of the amine fraction showed the presence of 3 moles of glutamic acid.

The analytical values indicate that the amine fragment arising from sulfurous acid cleavage of the fermentation *L. casei* factor is a tetrapeptide composed of 1 mole of *p*-aminobenzoic acid and 3 moles of glutamic acid. The theoretical elemental analysis for such a tetrapeptide is C—50.5, H—5.35, and N—10.69.

No significant amounts of compounds containing 1 or 2 carbon atoms could be detected in the sulfurous acid-inactivated solutions, suggesting that the carbon content of the fermentation *L. casei* factor was that represented by the degradation products.

In a summation of the data presented in this and the previous paper of this series, the following salient points serve as a guide to the formulation of the structure of the *L. casei* factor:

1. The fermentation *L. casei* factor can be degraded with alkali, under anaerobic conditions, to form the *dl* liver *L. casei* factor with the liberation of 2 moles of glutamic acid, thus establishing a direct relationship between the liver and fermentation *L. casei* factors.

2. Aerobic alkaline hydrolysis or sulfurous acid cleavage gives rise to a pteridine fraction and a primary aromatic amine. This indicates that the point of linkage is through the aromatic amine group to the pteridine.

3. 1 carbon serves as a linkage between the pteridine and the aromatic amine. This is indicated by the isolation of either or both the 2-amino-4-hydroxypteridine-6-carboxylic acid and the 2-amino-4-hydroxy-6-methylpteridine as degradation products in two methods of cleavage. Further indicative evidence is the inability to detect any 1 or 2 carbon-containing fragments in the hydrolysates.

4. The aromatic amine fraction arising from sulfurous acid cleavage is a tetrapeptide composed of 1 mole of *p*-aminobenzoic acid and 3 moles of glutamic acid.

5. The liver *L. casei* factor contains one glutamic acid in peptide linkage to the carboxyl group of the *p*-aminobenzoic acid.

STRUCTURE AND SYNTHESIS OF THE PTERIDINE DEGRADATION PRODUCTS OF THE FERMENTATION *L. CASEI* FACTOR

By John H. Mowat, James H. Boothe, Brian L. Hutchings, E. L. R. Stokstad, Coy W. Waller, Robert B. Angier, Joseph Semb, Donna B. Cosulich,* and Y. SubbaRow

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The two preceding papers of this series have indicated the nature of the degradation products obtained from the fermentation *Lactobacillus casei* factor[1]: namely, the *dl* liver *L. casei* factor, *p*-aminobenzoic acid, pyrrolidonecarboxylic acid, *l* (+) glutamic acid, 2-amino-4-hydroxy-6-pteridinecarboxylic acid,† and 2-amino-4-hydroxy-6-methylpteridine. The first four of these substances were known compounds and could be readily identified, whereas the two pteridine compounds were new substances which required further degradation, as well as synthesis, in order to establish their structure.

The above 2-amino-4-hydroxy-6-pteridinecarboxylic acid was first isolated from the oxidative alkaline hydrolysate of the fermentation *L. casei* factor. The empirical formula determined from analytical data, the ultraviolet absorption spectrum, the titration curve, and the positive test for guanidine, all of which have been described in the preceding papers, led us to suspect the presence of a pteridinecarboxylic acid. Decarboxylation of a few milligrams of the substance liberated a little less than one mole of carbon dioxide, and the residue, when purified, appeared to resemble 2-amino-4-hydroxypteridine, a substance which we had synthesized by reacting 2,4,5-triamino-6-hydroxypyrimidine with glyoxal. The synthesis of the 2-amino-4-hydroxy-6-pteridinecarboxylic acid was then effected by reacting 2,4,5-triamino-6-hydroxypyrimidine with ketomalonic ester, to give isoxanthopterin carboxylic acid[2] which was chlorinated with a mixture of phosphorus pentachloride and phosphorus oxychloride. The chlorine group was then replaced with hydrogen by reduction of the chloro com-

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† In this paper, we shall use the nomenclature and system of ring numbering recommended by "Chemical Abstracts" and the "Ring Index." Accordingly, the above 2-amino-4-hydroxy-6-pteridinecarboxylic acid could also be called 2-amino-4-hydroxy-6-pyrimido (4,5b) pyrazinecarboxylic acid. The same compound, when named according to a different system of numbering used in the German literature, could be called 2-amino-6-hydroxy-8-pteridinecarboxylic acid.

[1] **Hutchings, B. L., E. L. R. Stokstad, N. Bohonos, & N. H. Sloane.** Science **99:** 371. 1944.

[2] **Purrmann, R.** Ann. **548:** 284. 1941.

pound with hydrogen iodide. The product was found to be identical with the degradation product obtained from the fermentation *L. casei* factor. This substance was also synthesized by reacting 2,4,5-triamino-6-hydroxypyrimidine with ethyl-β,β-diethoxy-α-bromo-propionate.

By these procedures, the ring structure and the positions of the 2-amino group and the 4-hydroxy group were fully established. Furthermore, the synthesis of the compound from isoxanthopterin carboxylic acid indicated that the carboxyl group was very probably attached to the ring in the 6-position. This latter point, however, required further proof, since the structure of xanthopterin (and hence, the structure of isoxanthopterin carboxylic acid) had not been rigidly proved by Purrmann.[3]

Final proof that the carboxyl group was, indeed, in the 6-position was obtained by degrading the corresponding 2-amino-4-hydroxy-6-methylpteridine (which could be oxidized to the 2-amino-4-hydroxy-6-pteridinecarboxylic acid with alkaline potassium permanganate) by the method of Weijlard, Tishler, and Erickson.[4] The product from this degradation was identical with an authentic sample of 2-amino-5-methylpyrazine.

The 2-amino-4-hydroxy-6-methylpteridine, mentioned above, was prepared by decarboxylating 2-amino-4-hydroxy-6-pteridineacetic acid which was obtained by reacting 2,4,5-triamino-6-hydroxypyrimidine with methyl-γ,γ-dimethoxy acetoacetate in aqueous acetic acid. The 2-amino-4-hydroxy-6-methylpteridine was also shown to be identical with a methylpteridine obtained from the fermentation *L. casei* factor by sulfurous acid hydrolysis, as described in a preceding paper of this series.

The isomeric 2-amino-4-hydroxy-7-methylpteridine was prepared by reacting 2,4,5-triamino-6-hydroxypyrimidine with methyl glyoxal. Oxidation of this 7-methyl compound with alkaline potassium permanganate gave 2-amino-4-hydroxy-7-pteridinecarboxylic acid, isomeric with the acid obtained from the *L. casei* factor.

In studying the chemistry of the pteridines, it is often necessary to determine whether a side-chain is attached to the ring on the 6- or on the 7-position. In most cases, this information can be obtained by oxidizing the side chain with alkaline potassium permanganate and comparing the ultraviolet absorption spectrum of the product with the spectra of authentic samples of the 6- or 7-pteridinecarboxylic acids described above. Since the spectra of these acids differ markedly

[3] **Purrmann, R.** Ann. **546**: 98. 1940.
[4] **Weijlard, J., M. Tishler, & A. E. Erickson.** J. Am. Chem. Soc. **67**: 802. 1945.

from one another, and since the oxidation reaction usually offers no difficulty, it is possible to obtain the desired information rapidly and with the expenditure of only a few milligrams of material.

The identity and structure of the various degradation products having been clarified, it was then necessary to consider the linkages between them.

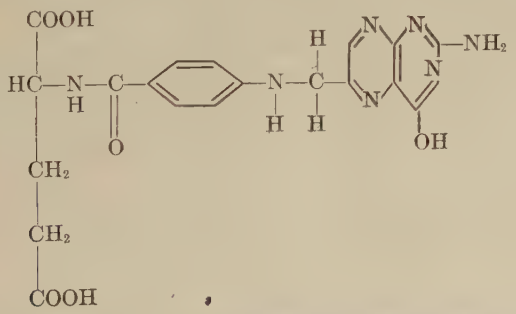
Hydrolysis of the pteridine-free aromatic amine fraction with the liberation of alpha amino acid nitrogen indicated that the carboxyl group of the *p*-aminobenzoic acid was attached to the glutamic acid through an amide linkage.

Hydrolysis of the fermentation *L. casei* factor, as described in the preceding papers, resulted in the simultaneous liberation of the aromatic amine fraction and a fluorescent pteridine fraction, indicating that the pteridine was attached to the amino group of the aromatic amine.

Since no pteridines having more than one carbon atom in the side chain could be isolated, and since no significant amounts of carbon dioxide, formaldehyde, formic acid, or other small fragments, could be detected in the hydrolysis mixtures, it seemed probable that the linkage contained only one carbon atom.

The marked stability of this linkage toward anaerobic hydrolysis in either acid or alkaline media, and the similarity between the cleavage of the fermentation *L. casei* factor and that of a simple model compound, *N*-benzyl-*p*-aminobenzoic acid, indicated that the pteridine and the *p*-aminobenzoyl glutamic acid were probably connected by a single methylene group.

On the basis of the above evidence, therefore, we postulated the structure of the liver *L. casei* factor to be:



N—[4—[[(2-amino-4-hydroxy-6 pteridyl)methyl] amino] benzoyl] glutamic acid.

SYNTHESIS OF PTEROYLGLUTAMIC ACID (LIVER *L. CASEI* FACTOR) AND PTEROIC ACID

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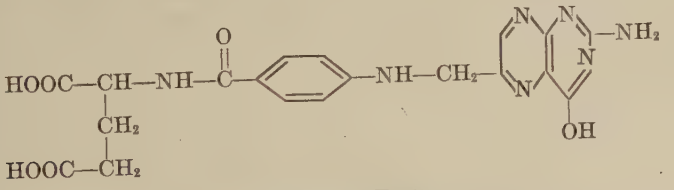
Upon completion of the degradation of the *Lactobacillus casei* factors and the synthesis of the fragments, the structure of the liver *L. casei* factor was proposed. The fermentation factor and the liver factor differed in the number of glutamic acid residues. The structure for the liver compound showed only one glutamic acid, while the fermentation factor appeared to contain three such residues. Both factors yielded *p*-aminobenzoic acid and the same pteridines upon degradation. On this basis, both factors appeared to have the same pteridine nucleus attached to the *p*-aminobenzoic acid, as indicated in the proposed structure for the liver factor. The chemical name is obviously too long for general usage. For the basic nucleus, a name indicating its pterin nature is desirable. Thus, the name "Pteroylglutamic Acid" is proposed for the liver *L. casei* factor. The fermentation *L. casei* factor and analogous compounds containing various amino acids can also be named as pteroyl derivatives. The basic structure for these compounds would, accordingly, be called "Pteroic Acid."

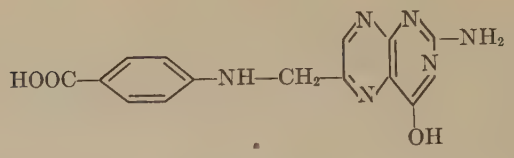
The syntheses of pteroylglutamic acid and pteroic acid are reported herein.



Liver *L. casei* Factor

N [4[[(2-amino-4-hydroxy-6-pteridyl)methyl] amino] benzoyl] glutamic acid

Pteroylglutamic Acid



4[[(2-amino-4-hydroxy-6-pteridyl)methyl] amino] benzoic acid
Pteroic Acid

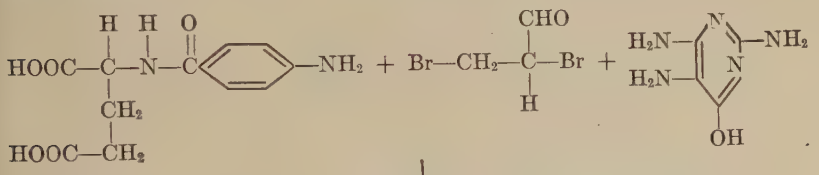
From an inspection of the proposed structure of pteroylglutamic acid, it was evident that a three-carbon compound was necessary for the synthesis of this structure from 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoylglutamic acid. In the previous paper, the pteridines were synthesized from 2,4,5-triamino-6-hydroxypyrimidine and a compound in which adjacent carbons contained functional groups capable of reacting with amines. By analogy, a reaction should occur between the 2,4,5-triamino-6-hydroxypyrimidine and a three-carbon compound in which the *p*-aminobenzoylglutamic acid was attached to a terminal carbon atom, and functional groups such as bromine or oxygen were on the other two carbons. α,β-dibromopropionaldehyde was the three-carbon compound chosen for this synthesis.

The first series of reactions in this synthesis was to combine the dibromopropionaldehyde and the *p*-aminobenzoyl compound, and then to react the product with 2,4,5-triamino-6-hydroxypyrimidine. When *p*-aminobenzoic acid or ethyl *p*-aminobenzoate and the dibromopropionaldehyde were reacted, crystalline products were obtained. The ease of hydrolysis of these compounds to *p*-aminobenzoic acid or ethyl *p*-aminobenzoate indicated that they contained anil structures. The remainder of the hydrolysis products were tars which could not be purified. Condensation of these anils with 2,4,5-triamino-6-hydroxypyrimidine gave little, if any, biologically active materials, as shown by assay against *S. faecalis* R. When *p*-aminobenzoyl-*l* (+)-glutamic acid was reacted with the dibromopropionaldehyde, a crude, hygroscopic, non-crystalline product was obtained. This also appeared to be an anil, because it hydrolyzed to *p*-aminobenzoylglutamic acid and a tar. Upon condensation of this anil with 2,4,5-triamino-6-hydroxypyrimidine, a very low yield of biologically active material was obtained.

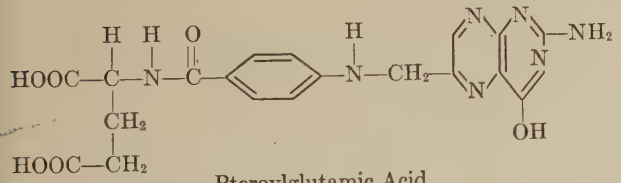
In view of the failure of the above reactions, the dibromopropionaldehyde was then reacted with the 2,4,5-triamino-6-hydroxypyrimidine, in an attempt to obtain a 6-bromomethylpteridine or a 6-hydroxymethylpteridine. From this condensation, a crude product was obtained and separated into two fractions. One fraction was identified as 2-amino-

4-hydroxy-7-methylpteridine. The other fraction could be characterized only to the extent that it was a 6-substituted pteridine, since it could be oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid.

In a final attempt to obtain pteroic acid derivatives by the use of dibromopropionaldehyde, equal molecular amounts of 2,4,5-triamino 6-hydroxypyrimidine and *p*-aminobenzoylglutamic acid were dissolved in water and treated with the dibromopropionaldehyde dissolved in an organic solvent. The yields were 30–50 per cent of crude material, containing 10–25 per cent pteroylglutamic acid. A series of experiments at various acidities showed that the best yields were obtained at pH 4. Buffering at pH 4 gave similar, but less consistent, yields than when the pH was controlled with alkalis. The addition of *p*-aminobenzoylglutamic acid to a reaction mixture of the 2,4,5-triamino-6-hydroxypyrimidine and the dibromopropionaldehyde gave much less pteroylglutamic acid than when all reactants were mixed together. The organic solvent used for the dibromopropionaldehyde made very little difference.



p-Aminobenzoylglutamic Acid

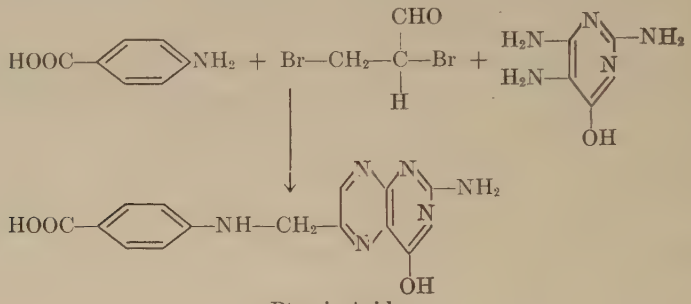


Pteroylglutamic Acid

The crude material was purified in the following way: It was dissolved in 0.2 N sodium hydroxide solution, at a concentration of 400 micrograms of active material per ml. Barium chloride was then added to 0.2 N, and the solution diluted with ethanol to a concentration of 20% by volume. The precipitate was filtered off and discarded. The solution was freed of excess barium, diluted to a concentration of 100 micrograms of active material per ml., and adjusted to pH 7. Again the precipitate was collected and discarded. The solution was then extracted three times with 10-volume portions of

butanol. The aqueous phase was then concentrated to 400 micrograms of pteroylglutamic acid per ml., and adjusted to pH 3 to precipitate this active material. Further purification was accomplished by dissolving this active material in 0.2 N sodium hydroxide solution, treating with charcoal, and precipitating at pH 3. Final purification was accomplished by recrystallization from hot water.

The pteroic acid was synthesized in the same way as pteroylglutamic acid, by substituting *p*-aminobenzoic acid for the *p*-aminobenzoylglutamic acid in the reaction where all the reactants were condensed at the same time. Pteroic acid possessed activity for *S. faecalis* R, but was inactive for *L. casei* and the chick.



Pteroic Acid

The crystallography, infra-red absorption, ultraviolet absorption, and biological activities for the liver *L. casei* factor and the synthetic pteroylglutamic acid have been compared and shown to be identical, in a previous publication.[1]

[1] **Angier, R. B.,** *et al.* Science **102:** 227. 1945.

DISCUSSION OF THE PAPER

Dr. J. J. Pfiffner (*Research Laboratories, Parke, Davis, & Company*):

I wish to take this opportunity, on behalf of my colleagues and myself, to congratulate the authors of this series of chemical papers on their success in accomplishing the complete proof of the structure of the liver *L. casei* factor. We have been working in this field for some time in the research laboratories of Parke, Davis, and Company, and I should like to make a few remarks on the matter of nomenclature. In following up the pioneering work of Hogan and his co-workers on the chick antianemia B vitamin, our group succeeded, several years ago, in isolating for the first time a pure crystalline compound from liver, which was an antianemia agent in the chick and which was also a growth factor for *L. casei*. We adopted the tentative designation, vitamin B_c, for this compound, since even at that early date there was considerable evidence that other growth factors for *L. casei* and *S. faecalis* occurred in nature. Proof that other such compounds did occur was offered shortly thereafter by Keresztesy, Rickes, and Stokes, of the Merck Laboratories, by their isolation of the SLR factor, which is not an antianemia agent in the chick. Some time ago, the Lederle Laboratories generously supplied us with a sample of the synthetic *L. casei* factor. We have com-

pared this product with vitamin B_c isolated from liver and from yeast digest and found them to be identical. Since the authors of the foregoing papers have demonstrated the structure of the compound and suggest the name *pteroylglutamic acid,* we propose to abandon the term vitamin B_c and adopt their name.

In this connection, it appears desirable to discuss briefly another interesting compound in this series, namely the crystalline chick antianemia factor, which we have isolated from yeast, but which is almost devoid of microbiological growth activity for *L. casei* and *S. faecalis.* This compound we had tentatively called vitamin B_c conjugate, since vitamin B_c (pteroylglutamic acid) is formed on hydrolyzing the conjugate with a specific enzyme or enzymes. We, in turn, had called the enzyme vitamin B_c conjugase. Whereas vitamin B_c (pteroylglutamic acid) contains one glutamic acid residue, vitamin B_c conjugate contains seven. In the system of nomenclature suggested by the authors, vitamin B_c conjugate is therefore pteroylheptaglutamic acid (more correctly, pteroylhexaglutamylglutamic acid). It follows that the enzyme, vitamin B_c conjugase, is a peptidase, and, since it does not hydrolyze vitamin B_c conjugate methyl ester, it can be identified as a pteroylglutamylcarboxypeptidase. The identification of the carboxypeptidase character of these enzymes is of particular physiological significance, since most of the pteroylglutamic acid in food exists in conjugated form. Some clinical observations by Dr. Bethell at the Simpson Memorial Institute and by Dr. Welch and Dr. Heinle at Western Reserve University point to an inability of pernicious anemia patients in relapse to utilize the yeast conjugate, as evidenced by absence of clinical response and failure to excrete the free vitamin following administration of the conjugate, in contrast to normal man who rapidly excretes free vitamin under the same conditions. The heptapeptide may, therefore, have unique value as an additional tool, throwing light on the etiology of certain of the clinical anemias.

PHARMACOLOGICAL STUDIES OF PTEROYLGLUTAMIC ACID

By Ben King Harned, Raymond W. Cunningham, Helen D. Smith, and Mary C. Clark

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Some of the acute and chronic effects of pteroylglutamic acid have been studied in mice, rats, guinea pigs, rabbits, cats, and dogs. The action of this substance is characterized by the lack of response to the usual pharmacological tests. It has a low acute and chronic toxicity and an almost complete absence of side reactions, even when the dose is far above the therapeutic range.[1, 2, 3]

EXPERIMENTAL

Materials

Although the data are recorded in terms of pteroylglutamic acid, the low solubility of the free acid required the use of a soluble salt for parenteral injections. In all experiments, except those on the kidney and in the guinea pig's skin, the sodium salt was administered in a solution of sodium bicarbonate. The preparation most frequently used was a 5 per cent solution made by dissolving the pteroylglutamic acid in 5 per cent sodium bicarbonate. The residual bicarbonate in this solution was 3 per cent. In each experiment, the control animals received a volume of 3 per cent sodium bicarbonate equivalent to the volume of the 5 per cent pteroylglutamic acid used. When more dilute solutions were employed, the sodium bicarbonate was reduced accordingly in the controls.

ACUTE TOXICITY

A few experiments with the oral and intraperitoneal administration demonstrated a very low toxicity, and as a result of these preliminary observations, intravenous administration was instituted and adhered to throughout the tests. The mortality count was made 14 days subsequent to the injection. Some of the deaths during this period may have been due to other causes, but no attempt was made to correct for this factor. The results are recorded in TABLE 1 and analyzed in FIGURE 1. An inspection of the data shows that the order of susceptibility to the compound is guinea pig, rabbit, rat, mouse. A

TABLE 1

THE ACUTE INTRAVENOUS TOXICITY OF PTEROYLGLUTAMIC ACID

Pteroylglutamic Acid in mgm. per kgm.		25	50	75	100	150	200	250	300	333	350	400	450	500	600	700	800
Mice*	No. Injected								18			18	18	18	18	4	9
	No. Dead								2			7	4	2	5	3	6
Rats*	No. Injected							8	20			36		28	10		
	No. Dead							0	2			14		14	4		
Rabbits*	No. Injected				4	4	4	5	4	2	3	3	3	3		3	
	No. Dead				0	1	1	2	1	1	2	1	1	2		2	
Guinea pigs*	No. Injected	4	4	4	4	4	2	2	1		2	1	5	2			
	No. Dead	0	0	2	1	2	2	1	1		2	1	5	2			

* The weight range of the animals used was: 18–22 grams for mice; 120–250 grams for rats; and 230–300 grams for the guinea pigs. One third of the rabbits weighed between 500 and 1000 grams, the other two thirds between 2200 and 2700 grams. The two groups were distributed throughout the range of doses. There was no obvious difference between the groups.

comparison of the species susceptibility at the $L.D._{50}$ indicates that the mouse can tolerate 5 times as much of the compound as the guinea pig. At the $L.D._{1}$, a similar comparison would give a ratio of approximately 10. The mortality curves for the mouse and the rat very closely approximate each other.

In the guinea pig series, 17 per cent of the non-survivors died during the first hour subsequent to the injection; 22 per cent during the first twelve hours; 28 per cent during the first forty-eight hours; 56 per cent during the first seventy-two hours; and 78 per cent during the first ninety-six hours. The data for the rabbits were similar. These delayed deaths, coupled with an inverse relationship between toxicity and water intake in the four species studied, suggested that renal damage might be an important factor. Pathological examination* confirmed this suggestion. A yellow substance, apparently pteroylglutamic acid, had been precipitated in the tubules.

In the four species studied, some of the animals died within thirty minutes after the injection. The percentage of these deaths was greatest in the rat series and least in the rabbit. Death followed a violent convulsion which was predominately tonic. Control experiments with sodium bicarbonate indicated that this substance was not an important factor in the deaths.

* The pathological examinations were made by Dr. F. I. Dessau.

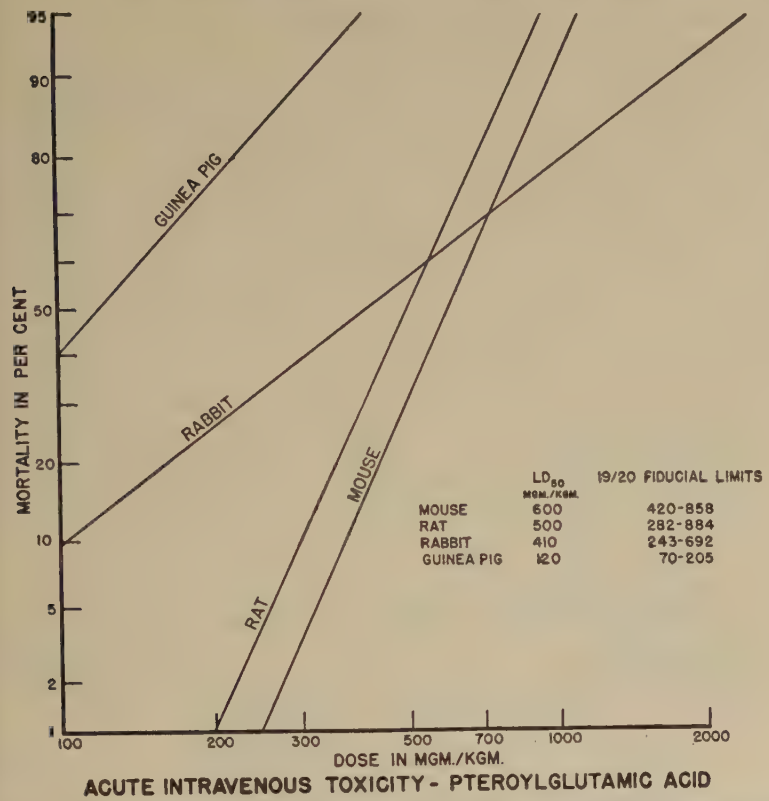

FIGURE 1. The experimental results were plotted on log probability paper[4] and straight lines fitted by eye.* The values for the L.D.50 were estimated from these lines. The 19/20 fiducial zones were estimated by a modification of the method of **Litchfield & Fertig.**[5] The modification allowed for the fact that the population was not homogeneous in all cases, and consisted in correcting the estimated values by multiplying by: $\sqrt{\frac{(Chi)^2}{n}}$, as described by **Wilcoxon & McCallan.**[4]

CHRONIC TOXICITY

Young male rabbits and rats were used in these experiments, and the pteroylglutamic acid was administered intraperitoneally, 5 days per week. In the first series of experiments, the daily dose given to rabbits and rats was 5 mgm. per kgm. The rabbits were dosed for 7 weeks and the rats for 10. The criteria for the comparisons were: survival, rate of growth, physical appearance, red blood cell counts, hemoglobin, total and differential white blood cell counts, and post-mortem examination. There was no difference between the treated and control groups. The growth curves are shown in FIGURES 2 and 3.

* We are indebted to Dr. Frank Wilcoxon from the Stamford Research Laboratories, American Cyanamid Company, for the analysis of the data on acute toxicity.

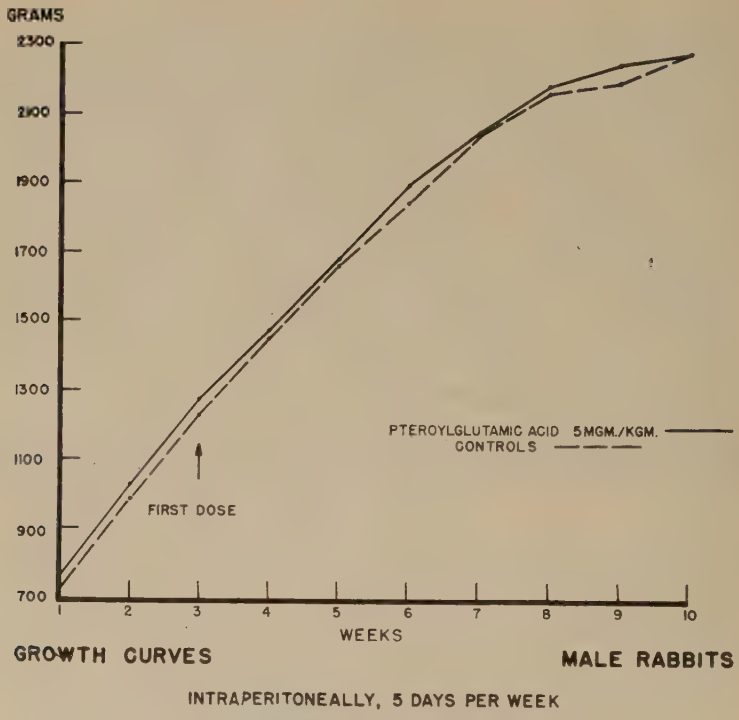

FIGURE 2. Each curve represents the average of fifteen rabbits.

When rabbits were given 50 mgm. per kgm. per day intraperitoneally for 10 weeks, there was a questionable retardation in growth (FIGURE 4), but between the treated and control groups there was no difference in the blood picture, the number of deaths, or the general appearance. However, the pathologist* reported that, at autopsy, the animals showed signs of renal injury probably due to tubular obstruction.

When rats were given 75 mgm. per kgm. per day intraperitoneally for 9 weeks, there was some depression of the growth curve (FIGURE 5). No other difference was observed during the dosing period, but again the pathologist* reported signs of renal injury probably due to tubular obstruction. It would be interesting to repeat these experiments and determine the effect of the period of dosing on longevity, but these doses were so far above the clinically effective range[1, 2, 3] that the work hardly seems justified.

* Dr. F. I. Dessau.

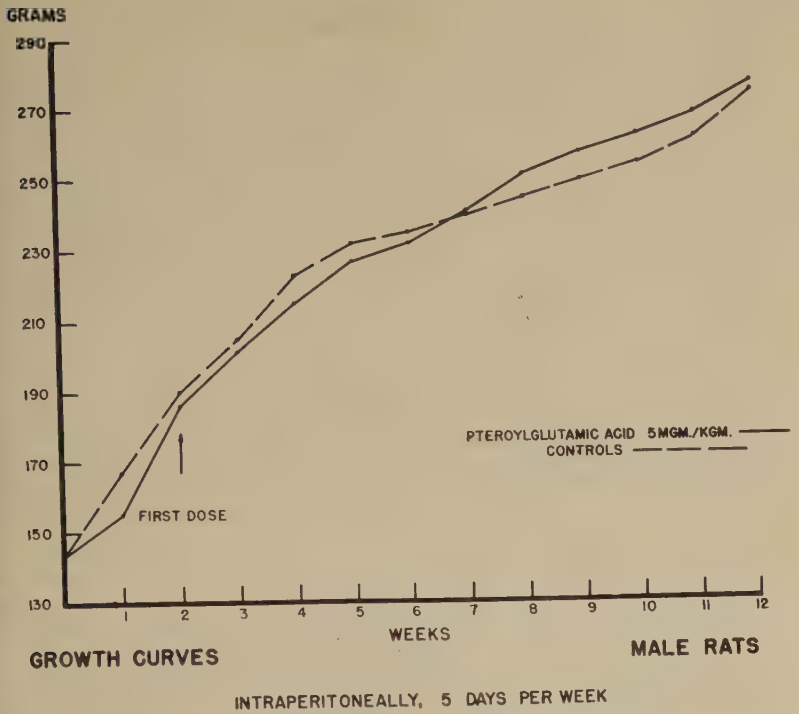

FIGURE 3. Each curve represents the average of fifteen rats.

RESPIRATION AND BLOOD PRESSURE

These tests were made on 6 dogs, 3 cats, and 1 rabbit. Except for 3 unanesthetized dogs, all of the animals were etherized. Typical experiments are presented in FIGURE 6. In doses of 1 to 100 mgm. per kgm. intravenously, pteroylglutamic acid does not affect appreciably the respiration of the dog or cat. The largest dose used on the rabbit was 50 mgm. per kgm., and this dose did not modify the respiration.

In both unanesthetized and anesthetized dogs, there was a temporary rise in blood pressure following the injection. The results were readily duplicated in the same and in different dogs. Five mgm. per kgm. increased the blood pressure 10 mm. of mercury; 20 mgm., 25 to 30 mm. of mercury; 40 mgm., 20 mm. of mercury; and 100 mgm., 10 mm. of mercury. Five minutes after the injection, 50 to 85 per cent of the pressor activity had disappeared, and in 30 minutes the blood pressure was always normal.

In two cats, one with an initial blood pressure of 84 mm. of mercury,

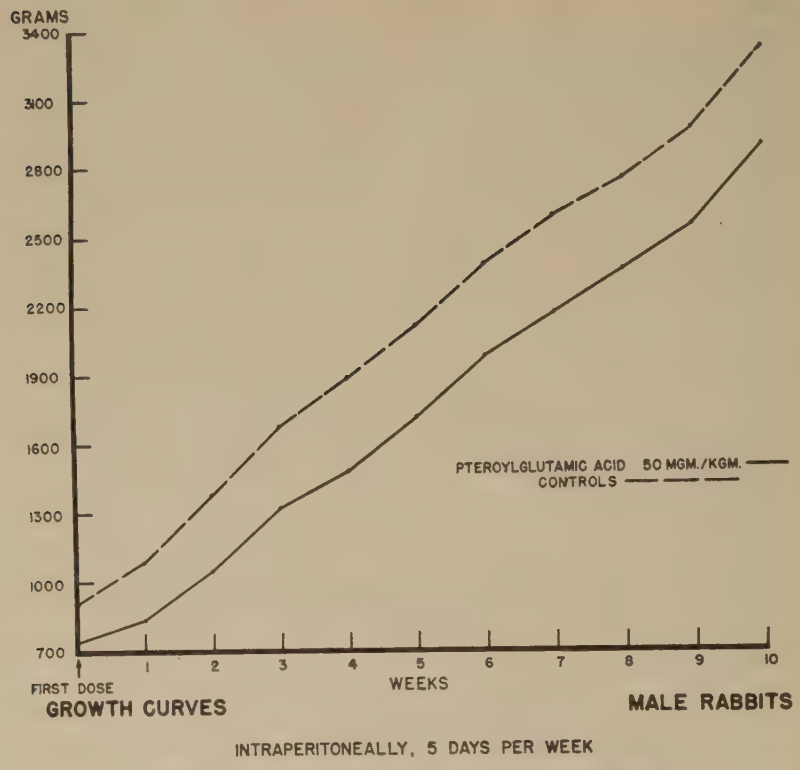

FIGURE 4. Each curve represents the average of eight rabbits.

and the other with 138 mm. of mercury, 20 mgm. per kgm. of pteroylglutamic acid produced a rise of 3 mm. of mercury in each. Forty mgm. per kgm. produced a rise of 2 mm., and 100 mgm. an elevation of 11 and 7 mm., respectively, in the two cats. A third cat with an initial blood pressure of 93 mm. of mercury responded to similar doses with a drop in pressure. Twenty mgm. per kgm. lowered the blood pressure 10 mm. of mercury, and 50 mgm. per kgm. lowered it 18 mm. of mercury. In each of the cats, the recovery time was rapid, approximately half as long as in the dog. The one rabbit used was given doses of 10 and 50 mgm. per kgm.; the response was similar to that observed in the first two cats.

ISOLATED INTESTINE

Doses of 1.0 to 10.0 mgm. of pteroylglutamic acid per 100 cc. of Tyrode's solution were tried on both spastic and normal strips of rabbit

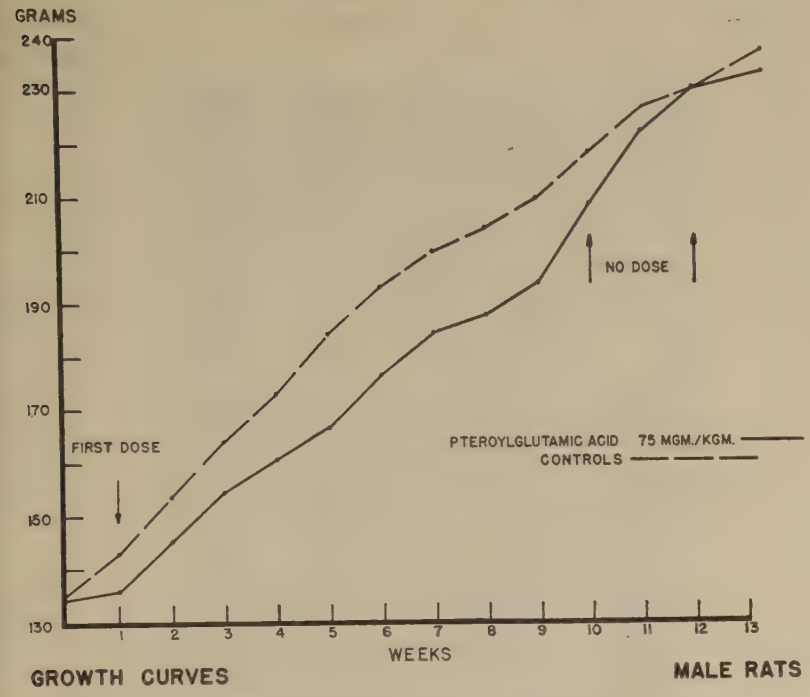

FIGURE 5. Each curve represents the average of twelve rats.

ileum. On the spastic strip, there was no action. On the normal strip, 1 mgm. produced no action, but 10 mgm. increased the tonus without changing the amplitude or rate of the contractions. This is a very low order of activity.

BLOOD SUGAR

Pteroylglutamic acid has no effect upon the blood sugar of fasted rats. The doses used were 50 and 100 mgm. per kgm., injected intraperitoneally. The results with the 100 mgm. dose are shown in FIGURE 7. On this figure are shown, also, the action of insulin and epinephrine, to demonstrate the quantitative response of the rats to hypoglycemic and hyperglycemic agents.

TESTS FOR IRRITATION

Repeated tests in guinea pigs, by the intracutaneous injection of 0.1 cc. of 1.5 per cent pteroylglutamic acid as the sodium salt, gave no evidence of irritation.

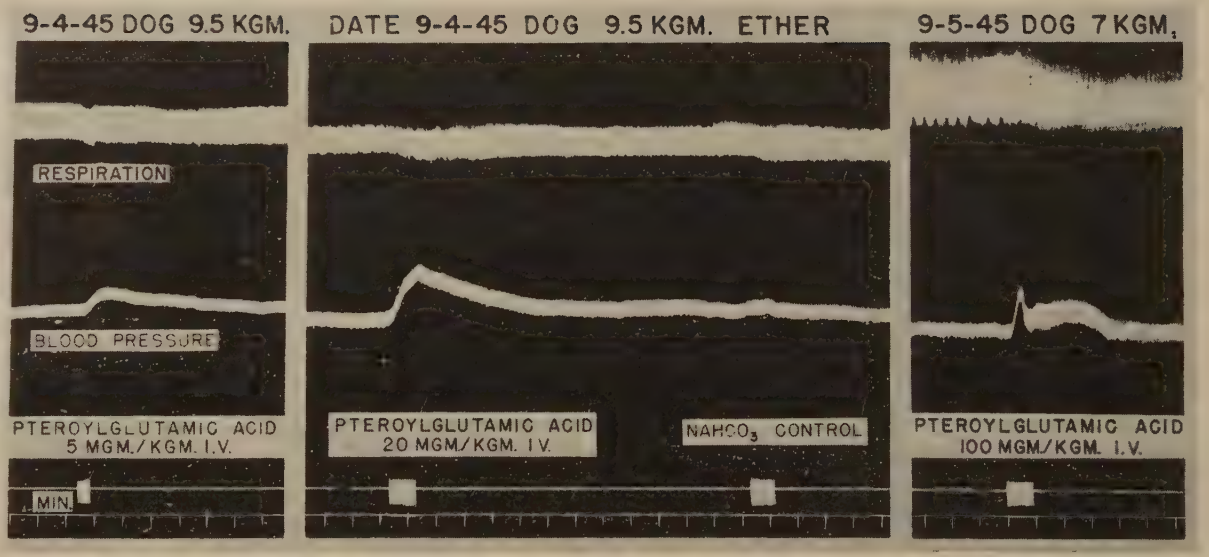

FIGURE 6. The time-record represents zero blood pressure. The blood pressure before each injection of pteroylglutamic acid was: section 1, 132 mm. of mercury; section 2, 126 mm. of mercury; section 3, 120 mm. of mercury.

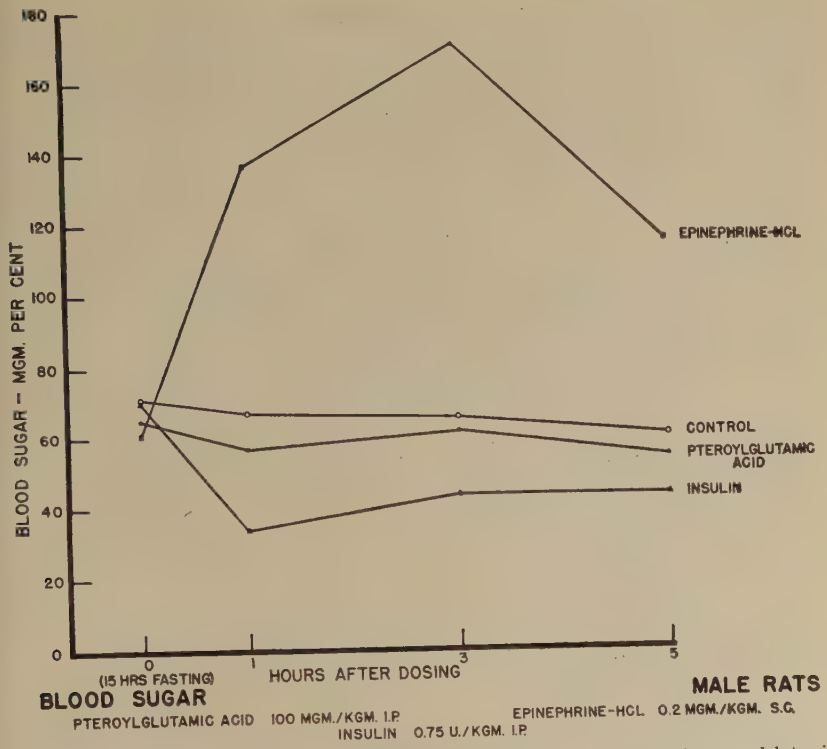

FIGURE 7. The number of rats in each group was: epinephrine, 2; control, 20; pteroylglutamic acid, 4; insulin, 4. The weights ranged from 150 to 250 grams.

KIDNEY

When 50 and 100 mgm. per kgm. of the free pteroylglutamic acid were given orally to rats in the Lipschitz test[6] for diuretic action, no effect was observed.*

SUMMARY

1. The pharmacology of pteroylglutamic acid is characterized by a lack of response to the usual pharmacological tests. It is not irritating when injected intracutaneously, it does not affect the blood sugar, and it has only a weak action on the isolated intestine. It does not affect the respiration, and the effects on the blood pressure are of a minor order.

2. In chronic experiments, the daily administration of 5 mgm. per kgm. intraperitoneally to rabbits and rats for two months produced no

* These studies were made by Dr. W. L. Lipschitz.

unfavorable reactions. In a similar period, daily intraperitoneal injections of 50 mgm. per kgm. to rabbits and 75 mgm. per kgm. to rats produced some changes in the tubules of the kidney, but no deaths.

3. The acute toxicity is low. Rats and mice tolerate approximately 200 mgm. per kgm. intravenously, with no evidence of action.

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PHYSIOLOGICAL ASPECTS

By Floyd S. Daft

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The isolation of pteroylglutamic acid ("*L. casei* factor," "folic acid," "vitamin M," "vitamin B_c") was announced in 1943.[1, 2] Adequate amounts of this new vitamin for physiological and nutritional tests did not become available, however, until synthesis was accomplished. This was announced in August, 1945,[3] less than a year ago. Great progress has been made, nevertheless, in our knowledge concerning the place of pteroylglutamic acid in human and animal nutrition. Evidence has been presented to indicate that this factor is a dietary essential for the chicken, the rat, the monkey, the guinea pig, the mouse, the dog, the turkey, and for man. Deficiency signs which have been reported for one or more species include: failure of growth, anemia, leucopenia, granulocytopenia, thrombocytopenia, poor feathering, diarrhea, gingivitis, necrosis of the gums, susceptibility to dysentery, achromotrichia, dehydration (as manifested by porphyrin-staining of fur and whiskers), a spastic type of cervical paralysis, a dermatologic syndrome, and perosis. Our knowledge concerning the biochemical lesion or lesions in pteroylglutamic acid deficiency is far from complete. Data have been accumulating which suggest that there is a defect, in deficient rats, in some phase of nitrogen metabolism. On the basis of results obtained in experiments with bacteria and in the clinic, it has been postulated that the role of this vitamin is to bring about the synthesis of thymine. This interesting suggestion deserves serious consideration, although it does not appear altogether probable, from preliminary tests with experimental animals, that lack of thymine is the sole defect in pteroylglutamic acid deficiency.

DEFICIENCY SIGNS

When the isolation of "vitamin B_c" or "*L. casei* factor" was announced by Pfiffner and associates,[1] and by Stokstad, Hutchings, and co-workers,[2, 4] it was noted by both groups of investigators that the pure vitamin was effective in preventing anemia and promoting growth in chicks. We, at the National Institute of Health, had the privilege of testing crystalline preparations from both laboratories in rats. The vitamin was administered to animals which had become leucopenic,

granulocytopenic, and anemic, while ingesting sulfonamides in a purified diet. Correction of the dyscrasias resulted, and growth, which had ceased, was resumed.[5] Campbell, Brown, and Emmett later reported[6] that deficient chicks became leucopenic and thrombocytopenic as well as anemic, and that these defects could be prevented by the inclusion of the crystalline vitamin in the experimental diet. Campbell and co-workers noted, also, that the vitamin appeared to be essential to the chick for normal feathering. In January, 1945, the monkey was added to the list of animals in which there is a demonstrated need for pteroylglutamic acid for normal hematopoiesis. Day and co-workers[7] observed that the administration to monkeys of a highly purified preparation of this vitamin was followed by complete remission of signs of "vitamin M" deficiency, which is characterized by loss of weight, leucopenia, granulocytopenia, anemia, bloody diarrhea, gingivitis, necrosis of gums, and susceptibility to dysentery. Reports of clinical trials of the new vitamin began to appear later in the same year. In September, Berry, Spies, and Doan reported that the administration of crystalline pteroylglutamic acid had a favorable influence on leucocyte equilibrium in malnourished patients,[8] and in October, Watson, Sebrell, McKelvey, and Daft presented data which suggested that concentrates of this factor may have been beneficial to patients with leucopenia following radiation therapy.[9] In November, Darby and Jones reported that two cases of sprue had improved markedly, following the parenteral administration of synthetic pteroylglutamic acid,[10] and Spies, Vilter, Koch, and Caldwell recorded the observation that patients with macrocytic anemia in relapse had shown significant hematopoietic responses to the same synthetic material.[11] Many clinical papers have followed, and it is now accepted that pteroylglutamic acid is beneficial in cases of sprue, pernicious anemia, and other macrocytic anemias. Further reports are awaited on the treatment of leucopenic patients.

In addition to these changes, a variety of other signs of pteroylglutamic acid deficiency has been noted in experimental animals. Before the pure vitamin was available, Martin observed that rats given sulfaguanidine (sulfanilylguanidine) in a purified diet became gray, and that the feeding of concentrates of "folic acid" restored the color of the hair.[12] Wright and Welch,[13] using succinyl sulfathiazole, corroborated these results and noted, further, that the sulfonamide-treated animals had porphyrin-caked whiskers and had also, despite a generous intake of pantothenic acid, a lowered hepatic level of this vitamin. The level of pantothenic acid in the liver could be raised to normal in these animals only by the administration of biotin and a

"folic acid" concentrate. Wright and Welch interpreted their results as indicating that biotin and "folic acid" are needed by the rat for the proper utilization of pantothenic acid. It was recently reported by Frost and co-workers that synthetic pteroylglutamic acid is also a chromotrichial factor for the chick.[14] Woolley and Sprince[15] have identified this vitamin as one of the growth essentials for the guinea pig, which they had previously called GPF-1; Nielsen and Black[16] have recorded the observation that it is a growth factor for the mouse; and Hertz and Sebrell[17] have demonstrated that it is needed by the chick for normal response to stilbestrol administration. From the data presented by Krehl and Elvehjem,[18] it appears that pteroylglutamic acid is needed by the dog. These investigators observed that recovery from blacktongue, following niacin therapy, was much more consistent in the case of dogs which were receiving a "folic acid" concentrate in their experimental diet than in the case of those which did not receive such a concentrate. A spastic type of cervical paralysis in turkey poults given deficient diets was observed by Richardson, Hogan, and Kempster,[19] who noted, also, that remission of symptoms followed treatment with crystalline "vitamin B_c." The successful therapy of a dermatologic syndrome in man with pteroylglutamic acid, has been reported by Coca.[20] Daniel, Farmer, and Norris[21] recently observed that perosis occurred in chicks on a diet low in "folic acid," and that the condition could be prevented by the administration of the synthetic vitamin.

The signs of pteroylglutamic acid deficiency which have been reported in the literature are listed in TABLE 1. In addition, Leuchtenberger, Lewisohn, Laszlo, and Leuchtenberger have made the interesting observation that this vitamin is a strong inhibitor of tumor growth in mice.[22]

METHODS USED FOR THE DEVELOPMENT OF SIGNS OF PTEROYLGLUTAMIC ACID DEFICIENCY IN EXPERIMENTAL ANIMALS

In most experimental animals which have been studied, signs of pteroylglutamic acid deficiency have developed simply as a result of the administration of an experimental diet which was deficient in this vitamin. In the rat, however, the application of this procedure has resulted in the appearance of blood dyscrasias in only a small percentage of animals. In one study involving 185 rats, Kornberg, Daft, and Sebrell[23] noted 6 instances of granulocytopenia. In order to obtain a

TABLE 1

REPORTED SIGNS OF PTEROYLGLUTAMIC ACID DEFICIENCY

Deficiency signs reported	Species
Failure of growth	Chick, rat, guinea pig, mouse, monkey
Anemia	Chick, rat, monkey, man
Leucopenia	Rat, chick, monkey, man
Granulocytopenia	Rat, monkey, man
Thrombocytopenia	Chick
Poor feathering	Chick
Diarrhea	Monkey
Gingivitis	Monkey
Necrosis of the gums	Monkey
Susceptibility to dysentery	Monkey
Achromotrichia	Rat, chick
Porphyrin-caked whiskers	Rat
Subnormal hepatic level of pantothenic acid	Rat
Subnormal response to stilbestrol	Chick
Subnormal response to niacin	Dog
Spastic type of cervical paralysis	Turkey poult
Dermatologic syndrome	Man
Perosis	Chick

higher incidence of blood dyscrasias in rats, investigators at the National Institute of Health have employed the six additional methods which are listed in TABLE 2. The second on the list, the administration of sulfonamides in purified diets, was the first method employed. Spicer, Daft, Sebrell, and Ashburn reported, in 1942,[24] that rats given sulfaguanidine or succinyl sulfathiazole in purified diets developed leucopenia, granulocytopenia, bone-marrow hypoplasia, and, occasionally, anemia. Kornberg, Daft, and Sebrell[25] later showed that rats given sulfadiazine or sulfathiazole developed similar lesions with a higher incidence of anemia. As noted in the section on "deficiency signs," sulfonamide-induced dyscrasias may be corrected by the administration of pteroylglutamic acid.[5] Of rats which have been given succinyl sulfathiazole, only about 10 per cent have usually become

TABLE 2

METHODS FOR DEVELOPMENT OF SIGNS OF PTEROYLGLUTAMIC ACID DEFICIENCY

(1) Deficient diets.
(2) Purified diets + sulfonamides.
(3) Purified diets + sulfonamides + controlled hemorrhage.
(4) Purified diets + thiourea + thyroxin (or thyroid powder).
(5) Pantothenic acid-deficient diets.
(6) Riboflavin-deficient diets.
(7) Protein-free diets.

anemic. It was shown by Kornberg and associates,[26] however, that when such animals were subjected to controlled massive hemorrhage, all became anemic, and that when bleeding was discontinued, there was a demonstrable failure in red cell regeneration. This failure could be prevented by the administration of pteroylglutamic acid. The fourth method for the development of blood dyscrasias in rats is the use of thiourea plus thyroxin or thyroid powder. As reported by Daft, Kornberg, Ashburn, and Sebrell,[27] rats which were given thiourea in a purified diet frequently became anemic and rarely granulocytopenic, while animals which were given, in addition, thyroxin injections or thyroid powder routinely became granulocytopenic and rarely anemic. Pteroylglutamic acid did not appear to affect the anemia, but, given in large doses for 10 day periods, it corrected the granulocytopenia.

The last three methods on the list ((5), (6), and (7) in TABLE 2) are similar to one another, in that the essential feature of each is the omission from a purified diet of a dietary essential other than, or rather in addition to, pteroylglutamic acid. The dietary ingredients omitted in these studies were pantothenic acid, riboflavin, and protein (casein), respectively. As reported by Daft, Kornberg, Ashburn, and Sebrell,[28] granulocytopenia, anemia, or both together, have been observed in pantothenic acid-deficient rats. The granulocytopenia, when alone, was corrected by pteroylglutamic acid, and the anemia, when alone, by pantothenic acid. When both granulocytopenia and anemia occurred together, both vitamins were needed for the rapid and consistent correction of either dyscrasia. Riboflavin-deficient rats, also, have become anemic, granulocytopenic, or both.[29] In these animals, pteroylglutamic acid corrected the granulocytopenia, and riboflavin, somewhat less consistently, the anemia. Neither in the experiments on pantothenic acid deficiency nor in those on riboflavin deficiency did the control animals develop blood changes. It appears from these data that a deficiency of pteroylglutamic acid has developed in rats deprived either of pantothenic acid or of riboflavin. A partial explanation of these results was obtained from paired-feeding experiments, in which one of each group of litter-mates was deprived of pantothenic acid[28] or riboflavin,[29] one was pair-fed a control diet containing adequate amounts of these vitamins, and a third was given the control diet *ad lib.* The data obtained indicate clearly that partial inanition, when purified diets are used, may result in granulocytopenia, and that this defect may be prevented or corrected by the administration of pteroylglutamic acid. Further progress resulted from the observation of Kornberg, Daft, and Sebrell that the feeding of protein-free or low-casein diets to

rats led to the development of granulocytopenia and anemia.[30] A study was made of the therapy of those animals which became granulocytopenic while receiving a protein-free diet. Insufficient data on the correction of anemia were obtained for conclusions to be drawn, but both protein (or the essential amino acids) and pteroylglutamic acid appeared to be needed for the correction of the white cell dyscrasia. From these results, it appears probable that the appearance of signs of pteroylglutamic acid deficiency in rats deprived of pantothenic acid, riboflavin, or protein, may be attributed to the lowered consumption of casein. It is not, as yet, clear why a decrease in the intake of this protein should have this effect, but a few of the possible explanations will be considered later in this discussion.

In the development and use of these seven methods, it appears that more questions have been raised than have been answered. These methods, nevertheless, constitute a valuable tool for further work.

It is interesting and perhaps significant that, although other factors are very evidently involved, pteroylglutamic acid has proved to be of therapeutic benefit to animals with blood dyscrasias developed in each of these seven ways.

PTEROYLGLUTAMIC ACID DEFICIENCY AND NITROGEN METABOLISM

Our knowledge of the function of pteroylglutamic acid in the bacterial cell and in the animal body appears to be, at best, somewhat fragmentary. It was demonstrated, several years ago, that thymine could substitute for this vitamin in the nutrition of lactic acid bacteria. Stokes has reported that "folic acid" could not be detected in *Streptococcus* cells grown in thymine medium, and has advanced the theory that this factor participates directly or indirectly as a coenzyme in the synthesis of thymine or a related compound in lactic acid *Streptococci*.[31] Spies, Vilter, Cline, and Frommeyer have successfully substituted thymine in large amounts for pteroylglutamic acid in the treatment of macrocytic anemias in relapse.[32] We have had sufficient thymine to treat only four granulocytopenic rats, two of which were anemic. Each was given 250 mg. of thymine during a 4-day period. The results were disappointing and suggest that a failure in the synthesis of thymine may not be the only biochemical lesion in pteroylglutamic acid-deficient rats. Further tests are necessary, however, before a definite conclusion may be drawn.

Wright, Skeggs, and Sprague[33] have reported that a diet high in

casein exerts a partial protective action in rats against the production, by succinyl sulfathiazole, of signs of nutritional deficiencies. Our results, which indicate that signs of pteroylglutamic acid deficiency may develop in animals whose intake of casein is restricted, have already been presented. In addition, we have been able to treat some of our granulocytopenic and anemic rats successfully, by increasing the percentage of casein in the diet. These data may indicate merely that casein is contaminated with pteroylglutamic acid. We have not, however, been able to demonstrate a sufficient degree of contamination to account for the results observed. It appears, on the basis of present information, that the possibility should at least be considered that, in deficient rats and other animals, there is a failure in some phase of nitrogen metabolism. This failure might lie in the synthesis of pteroylglutamic acid by intestinal bacteria or by the animal body, or in the normal metabolism of amino acids or related materials. The availability of adequate amounts of the synthetic vitamin and of a variety of methods for producing deficiency signs in experimental animals should be of great help in future investigations along these lines. It does not appear unreasonable to look to the near future for much new light on the precise physiological role, or roles, of pteroylglutamic acid.

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VITAMIN M DEFICIENCY*

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During the course of experiments designed to produce vitamin G (riboflavin) deficiency in the monkey, Day, Langston, and Shukers[1] discovered that the monkey (*Macacca mulatta*) is peculiarly susceptible to a fulminating disease whose characteristics are: leucopenia, anemia, thrombocytopenia, gingivitis, necrosis of the gums, loss of appetite, diarrhea, susceptibility to dysentery, and eventual death. Autopsy findings were ulcerated colon, liver damage, and adrenal changes[2, 3] Early work on this syndrome was reported in 1935. It was, at that time, recognized that the disease was due, not to a deficiency of any known vitamin, but probably to lack of an unknown nutrient, since it was preventable by supplementation of the experimental diet with certain liver fractions or yeast. In 1938, the factor was further differentiated, and tentatively named vitamin M.[3]

The factor was found to be present in liver, yeast, and certain liver fractions produced in the preparation of injectable liver extract. It is present, to a considerable extent, in material corresponding to Cohn fraction G,[4] but apparently is partly lost in further commercial fractionation of this material. Injectable liver extract, in a dosage adequate to maintain a pernicious anemia patient, does not protect a 3 kg. monkey indefinitely against vitamin M deficiency.[5]

Accumulated data on the deficiency syndrome may be summarized as follows:

Every animal which did not die too quickly of intercurrent disease developed leucopenia.

Of 62 experimental and control animals which developed leucopenia, 50 developed an anemia in which the red blood cell count fell below 4.0 million per cmm., for a protracted period. Of the twelve remaining animals, 2 on experimental diets lived longer than the average time required to develop anemia, and still failed to show significant lowering of red blood cell count and hemoglobin. One of these was an animal on the deficient diet supplemented with beef muscle, and the other received a liver-stomach preparation.

The average length of time on the experimental diet required to

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develop leucopenia (<10,000 WBC/cmm.) was 44 days; to develop anemia (<4.0$\overline{M}$ RBC/cmm.), 55 days. The length of time required to develop anemia appears to be directly correlated with the period required for the animal to become leucopenic. The dietary history prior to the experiment, if it has any effect, appears to affect equally the time requirement for development of leucopenia and anemia.

Because of the possibility that the *L. casei* factor and vitamin M might be related, microbiological determinations were made on vitamin M active substances. Data were obtained which indicated that there is a good correlation between the potential *L. casei* factor content, measured after appropriate enzyme treatment, of various vitamin M sources and their anti-leucopenia activity in the monkey.[6]

In collaboration with a group of Lederle Laboratory investigators, we have found the "fermentation" *L. casei* factor to be highly effective in vitamin M deficiency. Treatment of deficient animals with 2–4 mg. of this substance was followed by prompt relief of diarrhea and anorexia, rapid increases in white blood cell count, and reticulocyte showers, followed, some days later, by increases in red cell numbers and hemoglobin content of the blood.[7, 8]

Synthetic pteroylglutamic acid (liver *L. casei* factor)* has been tested in several deficient monkeys. Injection of 2 to 6 mg. of this material into 3–4.5 kg. animals with leucopenia was followed by dramatic leucocyte increases, reticulocyte responses, and, in those cases which survived, increases in red cell numbers. A prompt, but transient, increase in red blood cell count usually appeared within 24 hours and returned to pre-dosage levels in about three days. A more permanent increase followed several days later. The early increase may be due to a change in blood volume. Two of the 5 animals tested succumbed, even though, after treatment, granulocyte counts reached levels of 15,000 and 100,000 per cmm., respectively. Clinical improvement in the animals tested at this dosage level appeared less marked than with the fermentation factor. Data from a typical experiment are given in FIGURE 1.

* We are indebted to Lederle Laboratories for generous supplies of the synthetic vitamin.

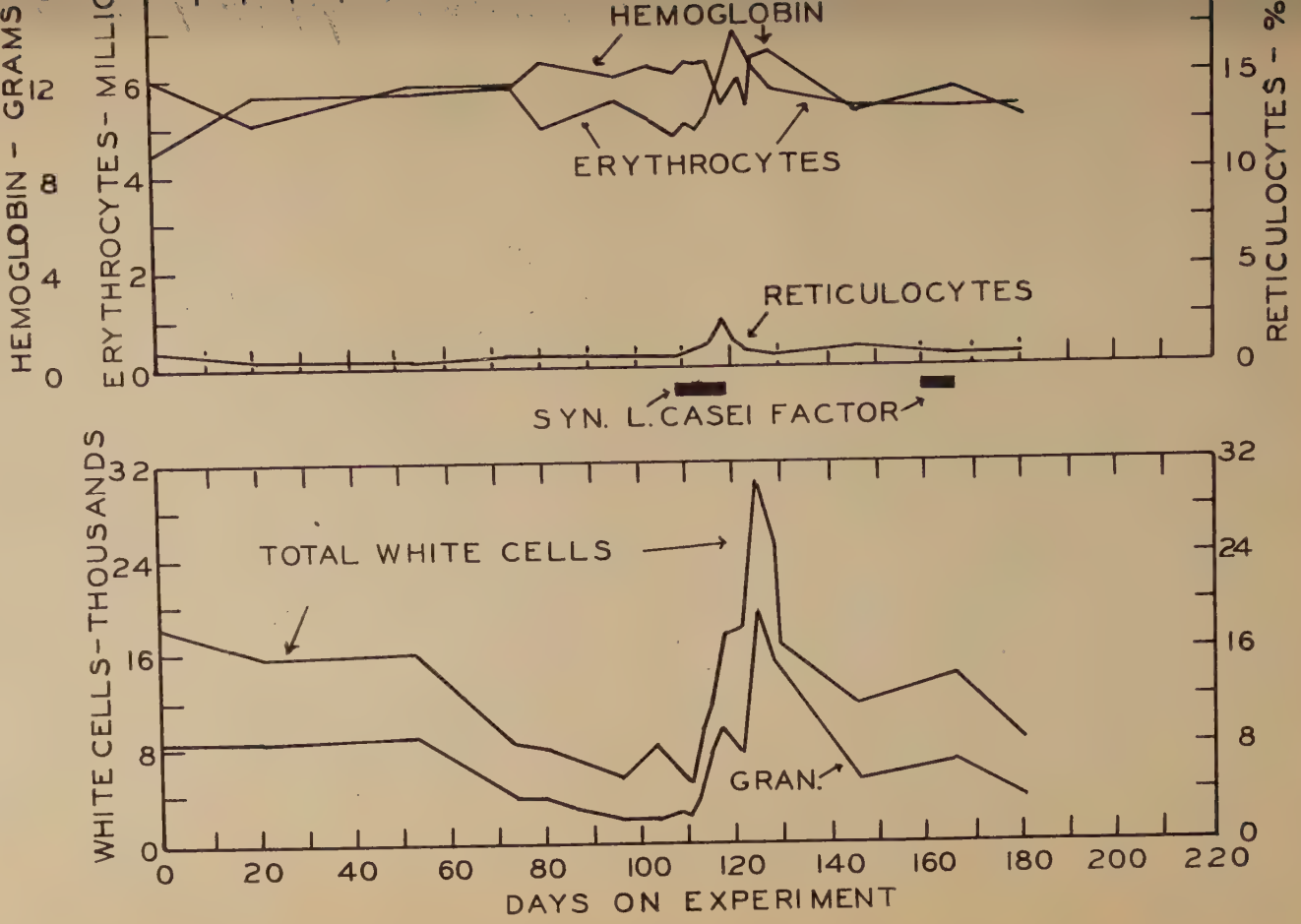
HEMOGLOBIN
ERYTHROCYTES
RETICULOCYTES
HEMOGLOBIN - GRAMS
ERYTHROCYTES - MILLIONS
RETICULOCYTES - %
SYN. L. CASEI FACTOR
TOTAL WHITE CELLS
GRAN.
WHITE CELLS - THOUSANDS
DAYS ON EXPERIMENT

FIGURE 1.

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SOME OBSERVATIONS ON THE THERAPEUTIC USEFULNESS OF SYNTHETIC *L. CASEI* FACTOR (FOLIC ACID)

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In the preceding papers, we have seen reports on some of the originally divergent research of many investigators, consummated in the synthesis of the *L. casei* factor. Only a few months ago, this substance was shown to be a safe and effective therapeutic agent in the treatment of sprue, pernicious anemia, and other related anemias.[1-8] Quickly, these findings were confirmed and extended by many investigators. The discovery of the therapeutic value of folic acid in these diseases is an event of major significance in the progress of medical science. For us, it marked a milestone in the progress of our studies on anemia which, with the assistance of many associates and collaborators, I have been carrying on for 15 years. In 1930 and 1931, I observed that many of our severely ill pellagrins had macrocytic hyperchromic anemia, which was cytologically indistinguishable from Addisonian pernicious anemia in relapse. That this anemia was not due to a lack of the intrinsic factor of Castle was demonstrated first by Spies, Payne, and Chinn,[9] who showed that the gastric juice of pellagrins, when incubated with ground beef, produces a factor capable of causing a remission in persons with Addisonian pernicious anemia. In 1935, Spies and Chinn[10] found that 63 per cent of their cases of severe pellagra had this type of anemia. This observation convinced me that, if we were to rehabilitate all the pellagrins who came under our care, we would have to look for effective methods of treating this type of anemia. Accordingly, we began our quest for substances which would cause remission of anemia, and began to establish a center where patients could be attracted and treated. Thus, when folic acid became available, we had many patients suitable for study.

During the past year, we have administered folic acid to 196 persons, under controlled conditions, and studied its effect on the blood and blood-forming organs. In 30 normal persons, 5 persons with iron

* University of Cincinnati Studies in Nutrition at Hillman Hospital, Birmingham, Alabama. The expenses of this study were borne by grants from the Edward Mason Williams Memorial Fund, Lederle Laboratories, Inc., and the Martha Leland Sherwin Memorial Fund.

deficiency anemia, 1 with anemia associated with carcinoma of the stomach, 6 with aplastic anemia, 5 with aleukemic leukemia, 6 with leukemia, 12 with post-infectious leucopenia, 3 with drug idiosyncrasy and 1 case of undiagnosed leucopenia, folic acid had no effect, and these cases will not be discussed further. The remaining 127 cases (74 with Addisonian pernicious anemia, 14 with nutritional macrocytic anemia, 4 with macrocytic anemia associated with pregnancy, 9 with macrocytic anemia associated with pellagra, and 26 with sprue) will be discussed in considerable detail.

In November, 1945, Dr. Carl F. Vilter, Dr. Richard W. Vilter, Virginia Hawkins, R.N., and I initiated our study to test the efficacy of folic acid in maintaining persons with Addisonian pernicious anemia. Each of the 24 patients chosen for this study had been maintained under our observation for a number of years on liver extract. We explained to the patients that we proposed to discontinue the liver extract and to give 30 mg. of folic acid each day, three times a week, if it were agreeable to them. Each one volunteered to cooperate in the study. After complete laboratory determinations and clinical examinations had been made, the first dose was given. The material has been taken in the presence of one of us on every occasion, and there has not been a relapse in any one of these patients. They feel well and many of them have gained weight. It is too early for us to state whether or not folic acid can replace liver extract in the maintenance of such patients, but this study is being continued and should eventually provide us with important information.

In the selection of all but the 24 cases of pernicious anemia to whom maintenance doses were administered, the following rigid criteria were used: (1) macrocytic hyperchromic anemia; (2) red blood cell count of 2.5 million or less; (3) color index of 1.0 or more; (4) megaloblastic arrest of the sternal bone marrow. An additional criterion for the selection of cases of Addisonian pernicious anemia was the absence of free hydrochloric acid, pepsinogen, and rennin in the gastric contents after histamine stimulation and, for the selection of cases of nutritional macrocytic anemia, the presence of free hydrochloric acid in the gastric contents after histamine stimulation. Additional criteria for the selection of patients with sprue were a flat glucose tolerance curve, "fatty" stools, and weight loss.

Ninety-eight of the patients were admitted to the hospital for preliminary observations, baseline determinations, and therapy. The other 5, who were treated as ambulatory cases, came to the hospital or clinic at frequent intervals throughout the study.

A detailed medical and dietary history was obtained, and a careful physical examination was made in each case. Throughout the control period and during the period of most active regeneration, red cell and white cell counts, reticulocyte counts, and hemoglobin determinations were made daily, using methods previously described.[5] Thereafter, these determinations were made less frequently. In every case, bone marrow studies were made prior to therapy and, in some cases, following therapy. The diet of all the hospital patients was rigidly controlled. Meat, meat products, and poultry were omitted. From one pint to one quart of milk daily was allowed. Bread, cereals, sugar, fats, vegetables, and fruits were permitted in amounts desired. The patients were always fed under the supervision of our staff, to insure their not getting any foods except those allowed on the diet. The 5 ambulatory patients ate their meals at home. No effort was made to control their diets, but frequent dietary histories taken throughout the course of this study revealed that their diets remained essentially the same as we had known them to be for many years.

At the time the study was initiated, each of the patients complained of loss of strength, vigor, and appetite. In most cases, there had been a considerable loss of body weight, and in the patients with sprue the loss had been from 20 to 30 per cent. The diarrhea in the patients with sprue was characterized by from five to twenty bowel movements daily. The stools appeared yellow or white, watery, foamy, and foul-smelling.

After baseline determinations were obtained, folic acid was administered parenterally in eight cases and orally in 95 cases. Two patients were given 20 mg., intravenously, daily; four received 50 mg., intravenously, daily; two were given 20 mg. intramuscularly, every day. To get the folic acid into solution for injection, we converted it into a soluble salt by adding normal sodium bicarbonate solution. To insure sterility, the solution was then passed through a Seitz filter. The 95 patients to whom it was administered orally were given daily doses ranging from 5 to 400 mg. When they were given 20 mg. or more, half of it was administered in the morning and half in the afternoon. It was administered in water suspension prepared by mixing it with four or five drops of cold water, and then adding 20 cc. of cold water, while the mixture was being stirred constantly. After the patient drank this material, a small amount of water was used to rinse the glass thoroughly, and he drank this also, to insure his getting as much of the folic acid as possible. During the latter part of the study, folic acid became available in tablets and was administered in this form.

On the fourth, fifth, or sixth day following the initiation of folic acid therapy, the reticulocytes began to rise, and peaked a few days later. This was followed by an increase in red blood cells and hemoglobin. The typical hemopoietic response of Addisonian pernicious anemia, nutritional macrocytic anemia, and sprue is shown in FIGURES 1, 2, and 3.

Soon after giving folic acid, the bone marrow changes. On the second or third day, an increased number of reticulocytes appears, and whole islands of regeneration form around the megaloblasts. Usually, by the fourth, fifth, or sixth day, the degree of change is tremendous. By this time, the reticulocytes are going up in the peripheral blood. It should be stressed that, in these cases, folic acid produces an increase in the red cells, the hemoglobin, the white cells, and platelets. As the remission becomes more advanced, the normoblasts increase in the bone marrow, and the megaloblasts decrease. Eventually, the bone marrow tends to approach normal.

The striking hemopoietic response is associated with a remarkable subjective improvement. Each patient voluntarily told us that he felt much stronger than he had for a long time, and that his desire for food had returned. The hospitalized patients, who, before treatment, had no desire to move, began to walk around the ward. Many of the ambulatory patients who had been driven to the clinic began to come on the streetcar, and some of them began to walk to the clinic. In many of the patients, particularly those with sprue and nutritional macrocytic anemia, loss of appetite and distaste for food had been so great that they had been eating less than five hundred calories daily. Three or four days after folic acid therapy was initiated, they began eating all the food offered and usually asked for more. It was not uncommon for them to eat from 3500 to 4500 calories daily. They gained about three pounds of body weight per week.

Of particular interest are our observations on the profound improvement in the alimentary tract symptoms of sprue, following the administration of folic acid. Within a few days following the initiation of therapy, the bowel movements, which had been occurring from five to twenty times daily, decreased to one or two daily. As therapy progressed, the color, odor, and consistency tended to become normal. Epigastric distress disappeared, and appetite returned. That intestinal parasitism is an almost constant finding in persons with tropical sprue, in Cuba, has been observed and reported by many investigators. As a special part of our collaborated study of sprue in Cuba,[11] the feces of 25 patients were repeatedly examined. We also examined the

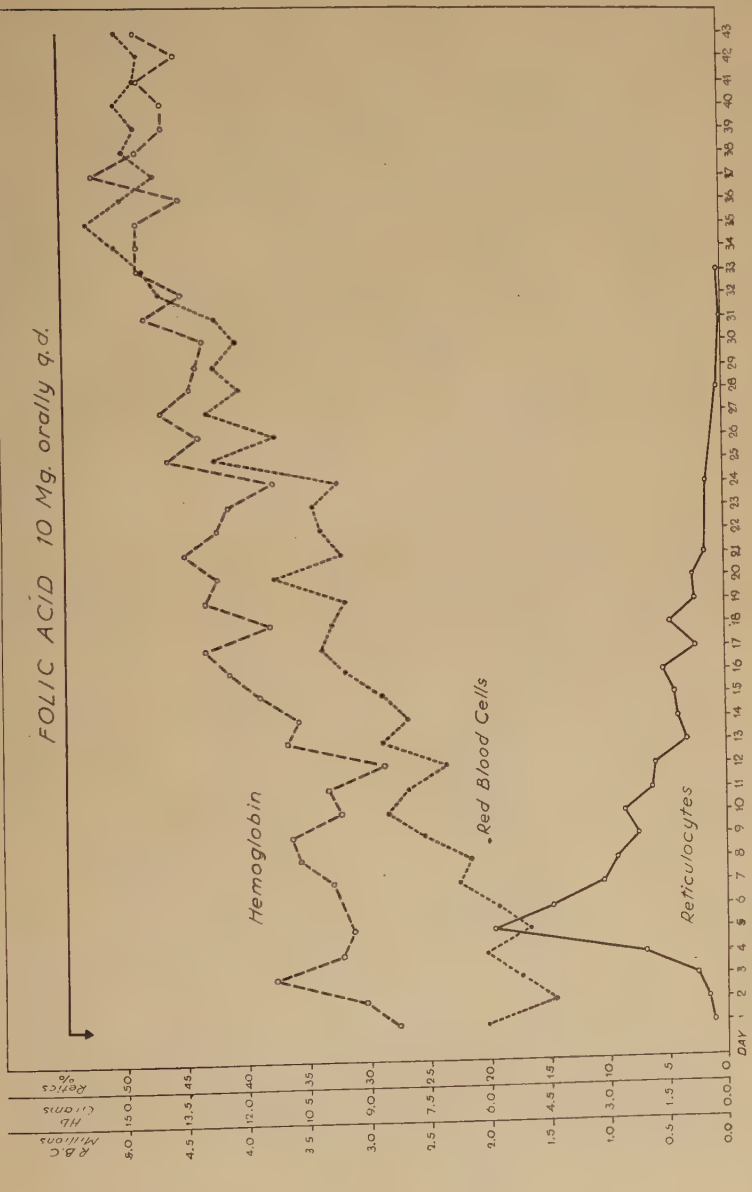
CASE OF PERNICIOUS ANEMIA - FOLIC ACID THERAPY
FOLIC ACID 10 Mg. orally q.d.
Hemoglobin
Red Blood Cells
Reticulocytes
R.B.C. Millions
Hb Grams
Retics %
DAY

FIGURE 1.

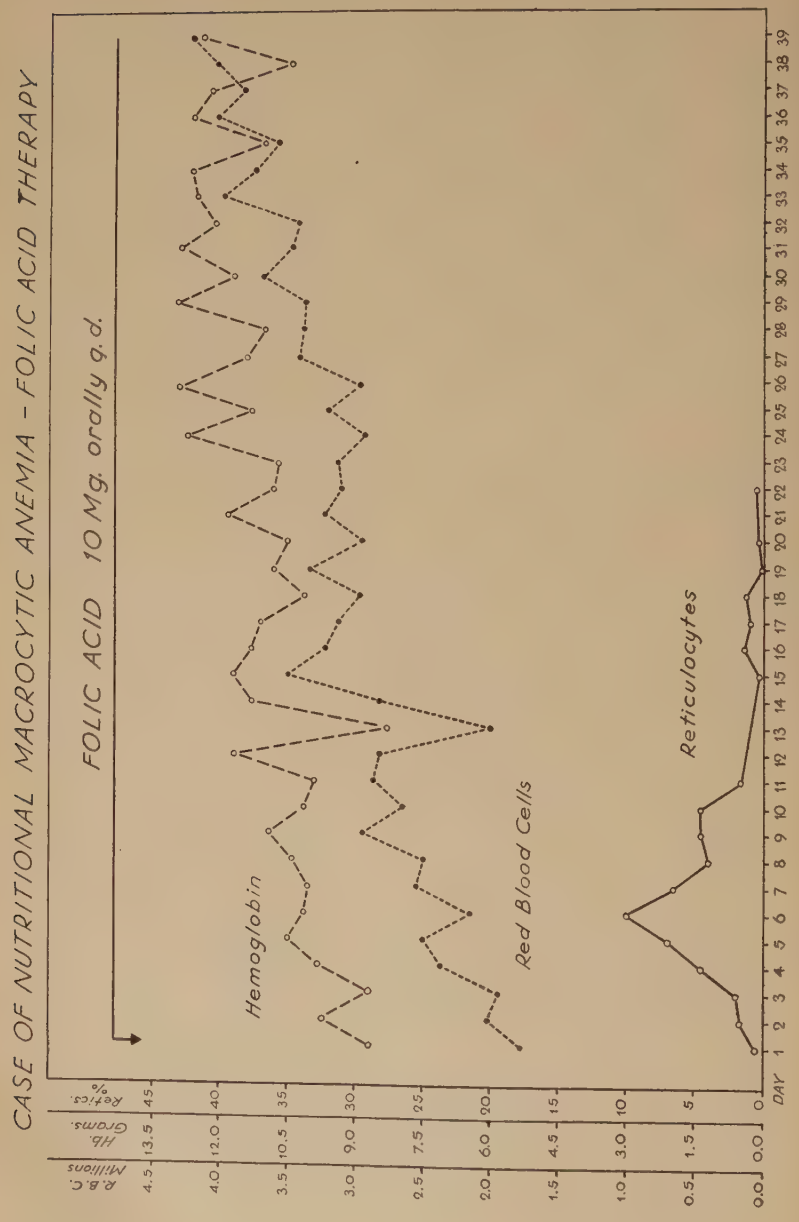
CASE OF NUTRITIONAL MACROCYTIC ANEMIA - FOLIC ACID THERAPY
FOLIC ACID 10 Mg. orally q. d.
Hemoglobin
Red Blood Cells
Reticulocytes
R.B.C. Millions
Hb. Grams.
Retics. %
DAY

FIGURE 2.

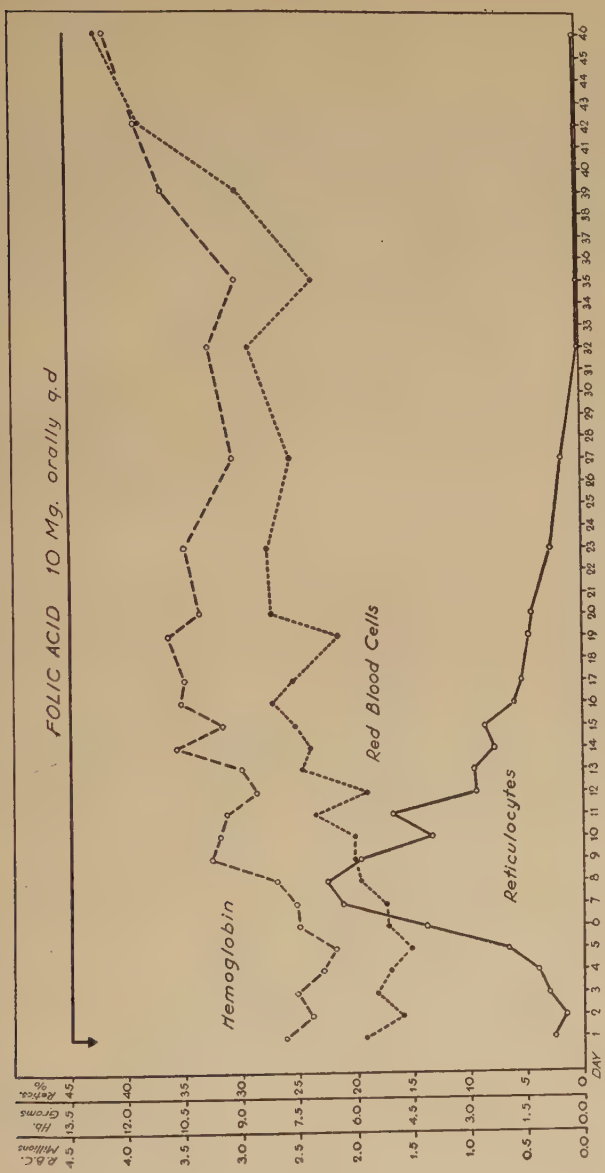
CASE OF SPRUE - FOLIC ACID THERAPY
FOLIC ACID 10 Mg. orally q.d
Hemoglobin
Red Blood Cells
Reticulocytes
R.B.C. Millions
Hb. Grams
Retics. %
DAY

FIGURE 3.

TABLE 1—FECES*

Case No.	Patient	Age	Sex	Parasites	Yeasts	Bacteria	pH
1	GG	29	F	*Trichuris trichiura* (ova)	Negative	*Staphylococcus* *Enterococcus* *Butyribacterium*	6.0
2	MS	45	F	*Trichuris trichiura* (ova)	Negative	*E. coli communior* *Butyribacterium*	4.5
3	CCB	48	F	*Trichuris trichiura* (ova) *Ascaris lumbricoides* (ova)	Negative	*E. coli communior* *E. coli acidi lactici* *Butyribacterium*	7.5
4	DL	67	F	Negative	Negative	*B. proteus* *Staphylococcus* *E. coli communior*	6.0
5	FG	31	F	*Trichuris trichiura*	*Monilia*	*Enterococcus* *E. coli communior* *Butyribacterium*	
6	RS	68	F	*Trichuris trichiura* (ova)	Negative	*E. coli acidi lactici* *Butyribacterium*	4.5
7	MI	33	F	Negative	Negative	*E. coli communior* *Aerobacter*	5.0
8	FM	52	M	*Trichuris trichiura* (ova)	*Monilia*	*E. coli communior* *Butyribacterium*	4.5
9	JG	40	M	Negative	Negative	*E. coli communior* *E. coli acidi lactici* *B. proteus* *Butyribacterium*	5.5
10	MR	70	M	Negative	*Hyphomycetes*	*B. proteus*	7.5
11	JC	63	M	*Endolimax nana* *Endamoeba coli* (cyst)	Negative	*E. coli communior* *E. coli acidi lactici* *Butyribacterium*	5.5
12	CC	44	M	Negative	*Hyphomycetes* *Monilia*	*E. coli communior* *Enterococcus* *B. proteus* *Butyribacterium*	4.5
13	ID	63	M	*Trichuris trichiura* *Necator americanus* *Strongoloides stercoralis* (ova and larvae)	*Monilia*	*Butyribacterium*	6.0

* Typical in 23 cases.

TABLE 1—FECES (*Continued*)

Case No.	Patient	Age	Sex	Parasites	Yeasts	Bacteria	pH
14	RCG	43	M	Negative	*Blastomyces hominis* (syst.)	*Pseudomonas* *Enterococcus* *E. coli communior* *Butyribacterium*	4.5
15	AF	75	M	*Endamoeba coli* (cyst) *Ascaris lumbricoides* (ova)	Negative	*B. pyocyaneus* *Enterococcus* *B. alcaligenes* *Staphylococcus*	6.5
16	FS	65	M	*Trichuris trichiura* *Necator americanus* (ova)	Negative	*E. coli communior* *Butyribacterium* *E. coli communior* *E. coli acidi lactici* *Butyribacterium*	5.5
17	PE	63	M	Negative	*Monilia*	*Klebsiella friedländeri*	4.5
18	GA	62	M	*Trichuris trichiura* (ova)	Negative	*Butyribacterium* *E. coli communior* *B. proteus* *Butyribacterium*	7.0
19	AM	37	M	*Trichuris trichiura* *Necator americanus* (ova)	Negative	*B. proteus* *Butyribacterium*	7.5
20	FP	66	M	*Trichuris trichiura* *Necator americanus* *Giardia* (ova and vegetative form)	Negative	*E. coli communior* *Enterococcus* *Butyribacterium*	4.5
21	EV	24	M	Negative	Negative	*E. Coli communior* *Staphylococcus* *Butyribacterium*	5.5
22	AF	46	M	Negative	*Blastomyces hominis*	Negative	6.5
23	AS	77	M	*Trichuris trichiura* *Necator americanus* (ova) *Chilomastix* (cyst)	*Blastomyces hominis*	*B. proteus* *E. coli communior* *Paracoli*	5.5
24	AH	41	M	Negative	Yeasts	*B. proteus* *E. coli acidi lactici*	7.5
25	AS	70	M	Negative	Negative	*E. coli acidi lactici* *Enterococcus*	6.0

large intestine, by curettage of the most altered portion of the mucosa of the rectum and sigmoid during rectosigmoidoscopy, and the small intestine by means of the Miller-Abbott tube, using the modified technique of Harris, and collecting samples of 3–5 cc. each from the jejunum, jejunoileal junction, and ilium. Each specimen thus obtained was examined microscopically while in the fresh, warm state. Immediately thereafter, a few drops of Lugol's solution were added to the specimen, and then microscopic examination was made, using the flotation method of Willis. Bacteriological culture was made on each specimen, according to "standard bacteriological technique." The individual specimens were planted on each of several different media, in an effort to obtain as many positive cultures as possible. The results of the microscopic examinations and of the cultures from the three sites mentioned above are shown in TABLES 1, 2, and 3.

We observed a conspicuous absence of parasites in samples obtained by curettage from the rectum and sigmoid. Intestinal parasitic ova, cysts, or adult forms were present in the feces in 56 per cent of the cases, and in the contents of the small intestines in 12 per cent of the cases. Bacterial culture revealed a multiplicity of organisms, most of which are known as the normal intestinal flora. We observed in the feces of 72 per cent of the cases a gram-positive, non-sporulating, anaerobic bacillus called *Butyribacterium*, which corresponds to the bacteroid of Castellani and Chalmers. Growth of cysts, of which *Monilia* formed the bulk, occurred in the feces of 40 per cent of the cases. The bacteria and yeasts were essentially confined to the stools, whereas the parasites occurred both in the small intestine and in the feces. The percentage of the occurrence of these organisms in samples obtained from the three sites studied is shown in TABLE 4.

Early in our studies concerning the effect of folic acid on the anemia of tropical sprue, we were impressed by the severity of the alimentary tract complaints in these patients and their improvement following folic acid therapy. Accordingly, we endeavored to determine what change, if any, occurred in alimentary tract function, when this substance was used as the only therapeutic agent.[12] Repeated gastrointestinal series were done on four persons, one normal person, and three patients with sprue. The three patients with sprue were on the same ward and received the same diet. One patient with sprue who received no therapy served as a control. The other two patients were given folic acid, and gastrointestinal series were done before, during, and after therapy. They were done at the same time intervals in the normal subject and in the control case of sprue.

TABLE 2

MATERIAL OBTAINED THROUGH RECTOSIGMOIDOSCOPY

Case No.	Patient	Age	Sex	Mucosa	Parasites	Yeasts	Bacteria
1	GG	29	F	Pale, Hypotrophic	Negative	Negative	Negative
2	MS	45	F	Pale, Hypotrophic	Negative	Negative	*B. proteus*
3	CCB	48	F	Pale, Hypotrophic, Sl. ong.	Negative	Negative	*Enterococcus* *E. coli acidi lactici*
4	DL	67	F	Pale, Hypotrophic	Negative	Negative	*B. proteus* *Staphylococcus*
5	FG	31	F	Pale, Hypotrophic	Negative	Negative	*E. coli acidi lactici* *Staphylococcus*
6	RS	68	F	Pale, Hypotrophic	Negative	Negative	Negative (mucous material)
7	MMI	33	F	Pale, Hypotrophic	Negative	Negative	*E. coli communior* *Enterococcus*
8	FM	52	M	Hyperhemic	Negative	Negative	*E. coli communior*
9	JG	40	M	Pale, Hypotrophic	Negative	Negative	*Staphylococcus*
10	MR	70	M	Pale, Hypotrophic	Negative	Negative	*E. coli communior* *B. proteus*
11	JC	63	M	Pale, Hypotrophic	Negative	Negative	*E. coli acidi lactici* *Butyribacterium* (fecaloid material)
12	CC	44	M	Pale, Hypotrophic	Negative	Negative	*B. proteus* *Staphylococcus*
13	ID	63	M	Pale, Hypotrophic	Negative	Negative	*E. coli communior* *Staphylococcus*
14	RCG	43	M	Pale, Hypotrophic	Negative	Negative	*B. proteus*
15	AF	75	M	Pale, Hypotrophic	Negative	Negative	*E. coli aerogenes* *Paracoli*
16	FS	65	M	Slight congestion	Negative	Negative	*E. coli acidi lactici*
17	PE	63	M	Pale, Hypotrophic	Negative	Negative	*E. coli communior* *B. proteus*
18	GA	62	M	Pale, Hypotrophic	Negative	Negative	*Enterococcus* *Butyribacterium* (fecaloid material)
19	AM	37	M	Pale, Hypotrophic	Negative	Negative	*E. coli communior*
20	FP	66	M	Pale, Hypotrophic	Negative	Negative	*B. proteus*
21	AF	46	M	Pale, Hypotrophic	Negative	Negative	*B. pyocaneus*
22	EV	24	M	Congestive	Negative	Negative	*Enterococcus* *E. coli communior*
23	AS	77	M	Pale, Hypotrophic	Negative	Negative	*B. pyocyaneus* *E. coli communior* *Paracoli*
24	AH	41	M	Hypotrophic	Negative	Negative	*B. proteus* *E. coli communior*
25	AS	70	M	Congestive	Negative	Negative	*B. pyocyaneus* *Staphylococcus*

TABLE 3—MATERIAL OBTAINED THROUGH THE MILLER-ABBOTT TUBE

Case No.	Patient	Age	Sex	Macroscopic findings	pH	Microscopic findings	Parasites	Yeast	Bacteria	Other data
1	CG	29	F	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious		Cholesterol and calcium carbonate crystals	Negative	Negative	Negative	
2	MS	45	F							3 trials, intubation not done
3	CCB	48	F						Negative	
4	DL	67	F	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	7 6.5 6	Calcium carbonate crystals	Negative	Negative	*Staphylococcus* *B. coliform*	
5	FG	31	F	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious Specimen IV, 8 ft., bilious	7.5 6.5 6 5.5	Calcium carbonate crystals	Negative	Negative	Resp. flora (*Streptococcus*) *M. tetragenus*	
6	RF	68	F	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	7.5 6.5 5.5	Calcium bilirubinate; phosphate; carbonate	Negative	Negative	Negative	
7	MI	33	F	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	6.5 7 6.5	Biliary pigm. Biliary salts	*Giardia* Specimen I, II, III	Negative	*M. catarrhalis* *Staphylococcus*	
8	FM	52	M							Trial, none
9	JF	40	M	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	4.5 4.5 5.5	Carbonate crystals	Negative	Negative	Negative	
10	MR	70	M	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	7.5 6.5 6.5	Bilirubinate; carbonate crystals	Negative	Negative	Negative	
11	JC	63	M	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	7.5 6.5 6	Idem	Negative	Negative	*B. coli*	
12	CC	44	M	Specimen I, 2 ft., bilious Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious	7.5 7 6.5	Idem	Negative	Negative	Resp. flora (*Streptococcus*) *M. catarrhalis*	
13	ID	63	M	Specimen I, 2 ft., bilious		Idem	Negative	Negative	Idem	

Case No.	Patient	Age	Sex	Macroscopic findings	pH	Microscopic findings	Parasites	Yeast	Bacteria	Other data
				Specimen II, 4 ft., bilious Specimen III, 6 ft., bilious						
14	RG	43	M	Specimen I, 2 ft., bilious		Idem	Negative	Negative	Negative	
				Specimen II, 4 ft., bilious						
15	AF	75	M	Specimen I, 3 ft., bilious	7.5	Idem	Negative	Negative	*Staphylococcus* *Streptococcus*	
				Specimen II, 6 ft., bilious	7.5					
16	FS	65	M							Trial, neg.
17	PE	63	M	Specimen I, 2 ft., bilious	7.5	Idem	Negative	Negative	*B. pyocyaneus* *B. alcaligenes* *B. fecalis*	
				Specimen II, 4 ft., bilious	7					
				Specimen III, 6 ft., bilious	6.5					
				Specimen IV, 8 ft., bilious	6					
18	GA	62	M							Trial, none
19	AM	37	M	Specimen I, 3 ft., bilious	7	Carbonate crystals	*Giardia* (veg. form) Specimen I, II, III		Negative	
				Specimen II, 5 ft., bilious	7					
				Specimen III, 8 ft., bilious	6					
20	FP	66	M	Specimen I, 4 ft., bilious	7	Negative	Specimen I negative Specimen II *Giardia* (veg. form) Specimen III *Necator* (ova)	Negative		
				Specimen II, 6 ft., bilious	6					
				Specimen III, 8 ft., bilious	6					
21	AF	46	M	Specimen I, 3 ft., bilious		Negative	Negative	Negative	Negative	
				Specimen II, 5 ft., bilious						
22	EV	24	M	Specimen I, 2 ft., bilious	7	Biliary pigm. and salts	Negative	Negative	*Staphylococcus* *B. proteus* *B. coliform*	
				Specimen II, 4 ft., bilious	6.5					
				Specimen III, 6 ft., bilious	6					
23	AS	77	M	Specimen I, 2 ft., bilious	7.5	Idem	Negative	Negative	*B. proteus* *E. coli communior*	
				Specimen II, 4 ft., bilious	6					
				Specimen III, 6 ft., bilious	7.5					
24	AH	41	M	Specimen I, 2 ft., bilious	7.5	Carbonate & bilirubinate crystals	Negative	Negative	*Staphylococcus* *M. catarrhalis* *Streptococcus* *B. coli*	
				Specimen II, 4 ft., bilious	6.5					
				Specimen III, 6 ft., bilious	6					
25	AS	70	M	Specimen I, 2 ft., bilious	7.5	Calcium carbonate crystals, oleagenous material	Negative	Negative	*B. coliform*	
				Specimen II, 4 ft., bilious	7					
				Specimen III, 6ft., bilious	6					

TABLE 4

PARASITES, YEASTS, AND BACTERIA

	Parasites	Yeasts	Bacteria
Feces	56%	40%	72% B B*
Curettage	0%	0%	8% B B
Miller-Abbott	12%	0%	0% B B

* *Butyribacterium*, gram-positive, non-sporulating, anaerobic bacteria, as described by Chalmers.

The profound changes which occur in the alimentary tract of persons with sprue, and the effect of folic acid therapy are shown in PLATES 1–7.

PLATE 1, taken 45 minutes after a patient with sprue received the barium meal, shows the barium column already broken. It shows the isolated, dilated segments and the "stack of coins" and "wheel" effects.

PLATE 2, taken in the same patient an hour after the barium meal, shows the striking distribution of barium into numerous, irregular-shaped clumps of barium which warrant the description "*moulage*" of barium. Several x-rays taken from 2 to 3 weeks later showed similar findings.

PLATES 3 and 4, taken in the same patient five weeks after the initiation of folic acid therapy, show the alimentary tract to be essentially normal. The continuous connecting barium column is neither fragmented nor interrupted.

PLATE 5, taken in another case of sprue, 45 minutes after the barium meal, shows the barium column already broken. The alternating intestinal spasm and dilatation are clearly shown.

PLATE 6, taken in the same patient only 15 minutes later, shows the abnormal gastrointestinal findings. Again, the barium is irregularly distributed in various parts of the small intestine, and there is considerable mucosal edema and spasm.

PLATE 7, taken in the same patient 34 days after the initiation of folic acid therapy, shows striking improvement, as evidenced by the continuous barium column, less mucosal edema, and less segmentation.

That persons with tropical sprue can be rehabilitated by the judicious use of folic acid is demonstrated by the fact that 18 of our patients, to whom it was administered, have been fully rehabilitated and have returned to work.[8] Following its administration, there was prompt blood regeneration, and the alimentary tract function tended to re-

turn to normal. PLATE 8 illustrates the stool of a patient, before and after folic acid therapy. Note the difference in color, texture, and volume. PLATES 9 and 10 are photographs of the same patient whose x-rays are shown in PLATES 1–4. PLATE 9 is a photograph taken prior to folic acid therapy; note the extreme pallor. PLATE 10 shows the same patient, after two months on folic acid therapy.

SUMMARY

1. The administration of synthetic folic acid to persons with Addisonian pernicious anemia, nutritional macrocytic anemia, and sprue in relapse is followed by profound blood regeneration. Strength and vigor return, and a rapid and spectacular gain in weight often follows. On the fourth, fifth, or sixth day, the reticulocytes begin to rise, and peak a few days later. This is followed by an increase in the red blood cells and hemoglobin, as shown in FIGURES 1, 2, and 3.

2. The hemopoietic response is associated with a great improvement in the altered alimentary tract function, as shown in PLATES 1–8. This improvement occurred in persons with intestinal parasitism, despite the fact that no anti-parasitic therapy was given, and that the parasites did not entirely disappear from the intestinal tract.

3. Already, many patients with nutritional macrocytic anemia, Addisonian pernicious anemia, and sprue have been rehabilitated (PLATES 9 and 10), following the administration of folic acid, and are now back at work.

4. While I realize that folic acid is not the major anti-anemia substance present in liver extract, our observations indicate that it is effective in producing a remission and in maintaining patients with Addisonian pernicious anemia, nutritional macrocytic anemia, and sprue. For persons who are sensitive to liver extract, I recommend it as a safe and effective substitute.

5. Twenty-four patients with Addisonian pernicious anemia, who had been maintained on liver extract for several years, have been given 30 mg. of folic acid three times weekly for seven months. Their blood values have remained essentially the same as they were on liver extract, and the patients state that they feel as well as when they were getting liver extract. These studies are being continued. I do not, at this time, recommend that folic acid replace liver extract in the maintenance of persons in the day-to-day practice of medicine. We have used it too short a time and know too little about its effect on combined system disease. The needed information will be procured in time.

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PLATES 1–10

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

PLATE 1

Patient with sprue, 45 minutes after barium meal, showing broken barium column, isolated segments, and "stack of coins" and "wheel" effects.

PLATE 2

Same patient as seen in PLATE 1, one hour after barium meal, showing *"moulage"* of barium.

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

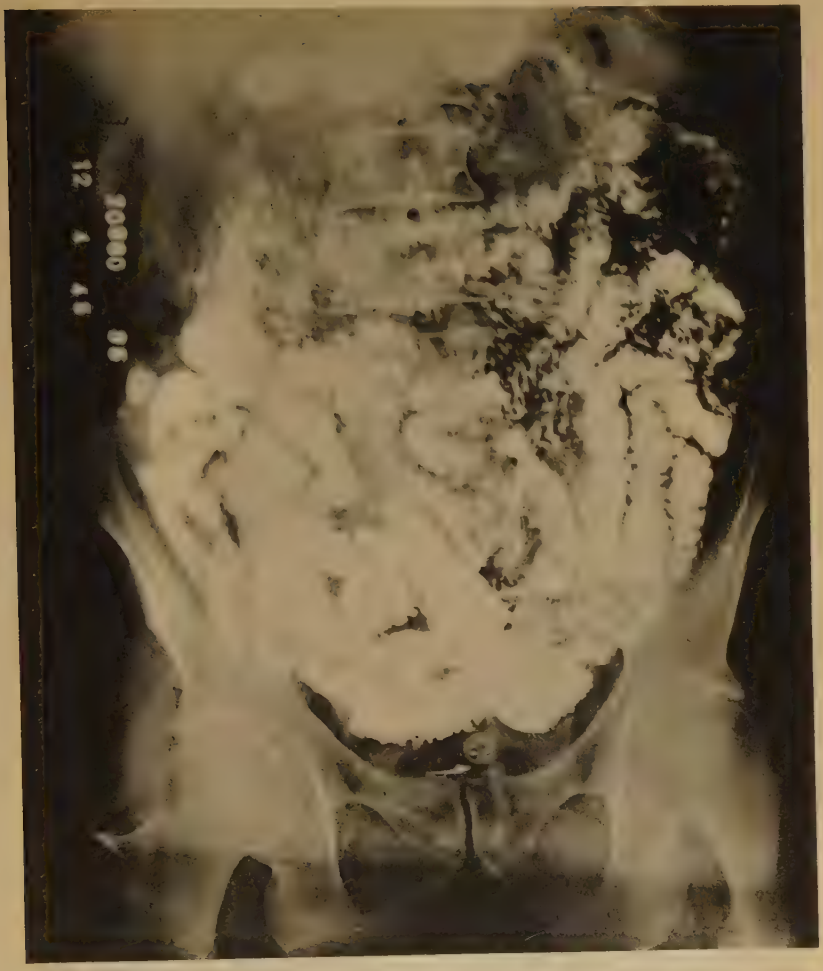
SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

PLATE 3

Same patient as seen in PLATE 1, five weeks after initiation of folic acid therapy, showing alimentary tract essentially normal.

PLATE 4

Same patient as seen in PLATE 1, five weeks after initiation of folic acid therapy, showing alimentary tract essentially normal.



SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

PLATE 5

Another patient with sprue, 45 minutes after barium meal, showing broken barium column and alternating intestinal spasm and dilatation.

PLATE 6

Same patient as seen in PLATE 5, 15 minutes later, showing barium irregularly distributed in various parts of small intestine, also mucosal edema and spasm.

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

PLATE 7

Same patient as shown in PLATE 5, 34 days after initiation of folic acid therapy. Note striking improvement evidenced by continuous barium column, less mucosal edema, and less segmentation.

PLATE 8

Stool of a patient before and after folic acid therapy. Note difference in color, texture, and volume.

PLATE 9

Photograph of patient whose x-rays are shown in PLATES 1–4, taken prior to folic acid therapy. Note extreme pallor.

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

PLATE 10

Photograph of same patient as shown in PLATE 9, taken after two months on folic acid therapy.

SPIES: THE THERAPEUTIC USEFULNESS OF FOLIC ACID

THE ROLE OF CONJUGATED AND FREE FORMS OF FOLIC ACID IN THE CONTROL OF PERNICIOUS ANEMIA*

I. CLINICAL OBSERVATIONS

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The ability of synthetic folic acid to produce remissions in patients with macrocytic anemia associated with megaloblastic arrest in the bone marrow has been established. This raises the question of why such patients should develop a deficiency of this substance. Folic acid is present in natural forms in many items of the daily diet, although in amounts unknown at the present time. Bacterial synthesis of folic acid, also, has been demonstrated to occur in the intestinal tract of animals, but whether this occurs in man has not been established. That folic acid is synthesized by normal human tissues has not been disproved, but no evidence for such a synthesis is available. It seemed possible, therefore, that some of the dietary forms of folic acid might not be effectively utilized in pernicious anemia.

This problem presented the necessity for studying naturally occurring derivatives of folic acid, including *Streptococcus lactis* R factor, "fermentation" folic acid, yeast conjugate, and other natural forms of folic acid which occur in the diet. *Streptococcus lactis* R factor has been shown to have no folic acid activity in any animals studied to date. Goldsmith has reported that "fermentation" folic acid was effective in one patient with macrocytic anemia. Castle, on the other hand, was unable to elicit a response in two patients with pernicious anemia who received 2.3 mg. and 3.6 mg., respectively, of "fermentation" folic acid, administered with normal human gastric juice. The yeast conjugate has been found to relieve folic acid deficiency in monkeys, chicks, and rats. It also occurred to us that other members of the vitamin B complex might contribute to the effect of folic acid.

The following brief case reports are presented to demonstrate the response of patients to folic acid and the failure of other components of the vitamin B complex to augment the effect of folic acid. (See TABLE 1 for summary of blood findings at beginning of treatment.)

* The work described in this paper has been supported, in part, by Lederle Laboratories, Inc.

The first patient, E. F., with pernicious anemia in relapse, received daily intramuscular injections of 1 mg. of synthetic folic acid, with a reticulocyte response of 10.5% on the seventh day. Addition of pantothenic acid (100 mg. daily, intramuscularly) did not produce a secondary response, during a subsequent period of 11 days, nor did the addition of all the known members of the vitamin B complex, plus biotin, *para*-aminobenzoic acid, choline, and inositol, produce any sec-

TABLE 1

LABORATORY FINDINGS IN THE BLOOD OF PATIENTS, PRIOR TO TREATMENT DESCRIBED IN TEXT

Patient	Erythrocytes millions per cmm.	Hemoglobin gm. per 100 cc.	Leucocytes per cmm.	Hematocrit	M.C.V.* c.μ	M.C.H.† $\gamma\gamma$	M.C.H.C.‡ per cent
EF	1.37	5.6	10,400	17	124.0	40.9	32.0
BD	1.19	5.1	1,800	16	134.5	42.8	31.8
JD	1.26	6.6	1,950	17	135.0	52.2	38.8
EH	1.28	5.1	5,200	16	123.0	39.8	31.8
LP	1.02	4.2	5,200	12	117.6	41.1	35.0
NB	1.49	5.0	1,750	15	100.5	33.6	42.4
LM	1.57	6.3	3,850	20	127.5	40.1	31.5

* = mean corpuscular volume.
† = mean corpuscular hemoglobin.
‡ = mean corpuscular hemoglobin concentration.

ondary response, during a third period. Erythrocytes, hemoglobin, and blood platelets increased in number throughout the periods of treatment. Leucopenia was never present in this patient.

Another patient, B. D., had a marked macrocytic anemia with a megaloblastic arrest in the bone marrow, induced by dietary deficiency associated with morphine addiction. Administration of daily intramuscular doses of 2.5 mg. of synthetic folic acid for 10 days produced a reticulocyte response of 18.9% on the fifth day. Addition of pantothenic acid (100 mg. daily, intramuscularly), during a subsequent 8-day period, failed to produce a detectable secondary response, as did addition of the known members of the vitamin B complex, plus biotin, *para*-aminobenzoic acid, choline, and inositol, during a third period of 10 days. Erythrocytes, hemoglobin, white cells, and platelets increased steadily throughout the test periods.

A patient, J. D., with pernicious anemia in relapse, received daily intramuscular injections of 1 mg. of synthetic folic acid. A reticulocyte peak of 13.2% occurred on the sixth day. Addition of known

members of the vitamin B complex, plus biotin, choline, inositol, and *para*-aminobenzoic acid, during a subsequent 10 day period, did not elicit a detectable secondary response.

A patient, E. H., with pernicious anemia in relapse, received daily intramuscular injections of 5 mg. of synthetic folic acid for 10 days, with a reticulocyte peak of 19.8% on the eleventh day. Addition of xanthopterin (50 mg. daily, by mouth), during a second period of 13 days, did not produce a detectable secondary response. During a third period of 10 days, the patient received daily intramuscular injections of 100 mg. of synthetic folic acid, with a questionable, small, secondary response. There was a rapid increase in erythrocytes, hemoglobin, blood platelets, and white cells.

A patient, L. P., with pernicious anemia in relapse, was of especial interest, because of failure to respond to synthetic folic acid as well as anticipated. During a first period of 10 days, she received 1 mg. of synthetic folic acid daily, intramuscularly, with a reticulocyte peak of only 4.2%. During a second period of 11 days, daily intramuscular injections of 6 mg. of synthetic folic acid produced a second, questionable response of only 3.0%. During a third period of 11 days, daily intramuscular injections of 12 mg. of synthetic folic acid produced a reticulocyte response of only 3.6%. During a fourth period of 10 days, daily injections of 12 mg. of synthetic folic acid were continued, and the patient received 36 mg. of xanthopterin daily, by mouth, in addition. A small, but clear-cut, reticulocyte response of 2.9% occurred during this period, suggesting that xanthopterin augmented the effect of the folic acid. Erythrocytes and hemoglobin were increasing slowly during this time. The platelets and white cells increased in number. During a fifth period, however, daily intramuscular administration of 15 units of purified liver extract (Armour) produced a prompt and theoretically maximal reticulocyte response of 13.2% on the fifth day of the period.

Lack of availability of the yeast conjugate delayed study of this substance until recently, when Dr. J. J. Pfiffner kindly supplied us with limited amounts. The prompt response of patients to synthetic folic acid suggested that the defect in hematopoiesis might reside in the inability of such patients to utilize conjugated forms of folic acid. That normal human gastric juice, fed together with beef muscle and other sources of "extrinsic" factor, will produce a hematopoietic response, is well known. It occurred to us, therefore, that conjugated forms of folic acid might be the "extrinsic" factor. Incubation of yeast conjugate with normal human gastric juice at pH 7.0 and at pH 4.5,

however, failed to cause any liberation of free folic acid (*L. casei* factor) from the yeast conjugate.

This was also tested in two patients with pernicious anemia. The first patient, N. B., received 1 mg. of yeast conjugate by mouth daily, for 10 days, with no response. During a second period of 11 days, 100 cc. of normal human gastric juice were administered with the yeast conjugate, again without response. During a third period of 11 days, the patient received a daily oral dose of 0.35 mg. of synthetic folic acid, an amount equivalent to the conjugate administered previously, since yeast conjugate has a molecular weight 2.8 times that of synthetic folic acid. On the tenth day, a small, but undeniable, reticulocyte response of 4.2% occurred. Subsequent daily treatment with 5 mg. of synthetic folic acid by mouth produced a reticulocyte response of 14.2%, with rapid rise in erythrocytes and hemoglobin.

The second patient, L. M., received daily intramuscular injections of 0.85 mg. of synthetic folic acid, during a first period of 10 days, with a delayed and small reticulocyte response of less than 3.0%. During a second period of 12 days, she received daily intramuscular injections of 2.5 mg. of yeast conjugate, a dose equivalent to the preceding treatment with synthetic folic acid, again based on the fact that yeast conjugate has a molecular weight 2.8 times that of synthetic folic acid. There was no reticulocyte response. That no response occurred indicated only that the conjugate was no more active than an equivalent amount of the synthetic folic acid. The patient was then given the entire remaining amount of conjugate available, 30 mg., in a single dose. No reticulocyte response occurred. After 12 days, a single equivalent dose of 11 mg. of synthetic folic acid was given intramuscularly, and a prompt reticulocyte response of 6.1% occurred. Subsequent daily intramuscular doses of 20 mg. of synthetic folic acid caused a theoretically maximal reticulocyte response of 26.8% to occur.

It was demonstrated in both of these patients, therefore, that the yeast conjugate was not utilized, whereas an equivalent amount of synthetic folic acid produced a hematopoietic response. In the first patient, further, addition of normal gastric juice did not enable the patient to respond to yeast conjugate, indicating that yeast conjugate has no extrinsic factor activity.

II. BIOCHEMICAL ASPECTS

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In addition to the failure of conjugated folic acid of yeast to produce a clinical response in pernicious anemia, there is other evidence of a biochemical defect in the utilization of this material. Observations on the excretion of the *L. casei* factor have disclosed that only traces appear in the urine of normal individuals on ordinary diets; this excretion approximates 2 to 4 micrograms per day. When synthetic folic acid (*L. casei* factor) is administered parenterally, from 15 to 75% of the dose usually appears in the urine within 24 hours.

In the patient given daily intramuscular injections of 2.5 mg. of vitamin B_c conjugate, there was no appreciable augmentation of the basal urinary elimination of the *L. casei* factor; of the injected doses, in terms of *L. casei* factor, only 1%, on the average, appeared in the urine daily. On the previous dosage regime, 0.85 mg. of synthetic folic acid per day, the patient had excreted approximately 15% of the daily dose. Following the administration of the single intramuscular dose of 30 mg. of conjugate, none of the material was found in the urine, either in the free or in the conjugated form. However, a dose of the equivalent amount (11 mg.) of synthetic folic acid not only caused a clinical response, but also resulted in the excretion of approximately 4.1 mg. of the *L. casei* factor during the following 48 hours.

It is clear, therefore, that, in these patients with pernicious anemia, the conjugated folic acid of yeast is not broken down appreciably to release *L. casei* factor, and its failure to appear as such in the urine suggests that it is either destroyed or, more probably, stored in the tissues.

Although conjugated folic acid appears to be utilized effectively by chicks, rats, and monkeys, it is necessary to offer proof that human subjects without pernicious anemia can make use of such materials. Since a patient with a purely nutritional deficiency of folic acid was not available, the ability of a normal subject to metabolize the yeast conjugate was demonstrated by a study of the urinary excretion of the *L. casei* factor, following dosage with the conjugate. A normal human subject, whose output of *L. casei* factor in the urine was known consistently to

approximate 3 micrograms per day, was given intramuscularly 800 micrograms of synthetic folic acid on each of two successive days. Of the material injected, 10 and 22 per cent, respectively, was excreted in the urine, with a prompt return of the urinary output to the basal level, 3 micrograms, on the following day. The subject was then given intramuscularly an approximately equivalent amount of yeast conjugate, 2800 micrograms, daily for two days. These injections resulted in the appearance in the urine of 84 and 82 micrograms of the *L. casei* factor, 8.4 and 8.2 per cent, respectively, of the theoretical amounts capable of being released from the conjugate.

It is evident that the conjugases of the normal human subject, in contrast to those of the patient with pernicious anemia, released considerable amounts of folic acid from parenterally administered yeast conjugate.

A logical deduction to be made from these data might be that the anti-pernicious anemia factor is concerned with the liberation of folic acid from its naturally occurring conjugates, and that the failure to maintain adequate hematopoiesis on the normal dietary intake of folic acid-containing substances is due to a deficiency in the utilization of the conjugated materials, which *are* utilized by normal human subjects.

Many experiments have been conducted *in vitro* on the conjugate content and conjugase activity of the sternal bone marrow of patients with pernicious anemia and of normal human subjects. The conjugase activity of this material, at pH 4.5, but not at pH 7.0, on some occasions, has been augmented as much as 2½-fold by the addition of sufficient 15-unit liver extract (Lederle) to supply about 0.1 unit, or approximately 0.7 mg. of total solids; smaller amounts have not yet been studied. Although this effect of liver extract *in vitro* has been observed in several instances, the phenomenon is not regularly observed, and no definite claim for its significance can now be made. Conceivably, liver extracts may contain a factor concerned with the function of certain conjugases, either directly (prosthetic group or co-factor) or indirectly (by influencing the rate of removal of an inhibitory effect on the enzyme). Although the evidence now available can only be considered to suggest this possibility, preliminary observations indicate that the injection of highly purified liver extracts, containing less than 0.1 microgram of folic acid per unit of anti-pernicious anemia activity, cause a slight, but definite, increase in the urinary excretion of *L. casei* factor. This effect of liver extracts has not been observed in normal subjects.

From the experiments described, it is not possible to conclude that

the so-called pH 4.5-conjugase is deficient in pernicious anemia. However, in conjunction with the failure of the yeast conjugate to produce any measurable clinical response in the doses used, or to be excreted in the urine of pernicious anemia patients, either as free or as conjugated *L. casei* factor, these findings *in vitro* support the concept that a biochemical defect in the metabolism of conjugated folic acid exists in pernicious anemia. Should further studies of various fractions of liver indicate a definite relation of the anti-pernicious anemia factor to the function of the conjugase studied at pH 4.5, a means for the assay *in vitro* of the anti-pernicious anemia activity of liver extracts might be afforded.

Whether this possible role of the anti-pernicious anemia factor is its only function, cannot now be stated. In patients who responded poorly to parenterally administered synthetic folic acid, it is conceivable that folic acid was excreted with abnormal rapidity, or was inactivated to an extent greater than in more responsive cases. However, the effectiveness of liver extract in one folic acid-refractory patient suggests that factors of liver extract are also concerned with functions other than the release of folic acid from its conjugates.

In opposition to the attractive hypothesis that the anti-pernicious anemia factor is concerned with the synthesis of folic acid in the body, is the fact that very large doses of anti-pernicious anemia factor are quite ineffective in the folic acid-deficient monkey (Day and coworkers), rat (Daft and Sebrell), and chick (Stokstad and Jukes). That folic acid is a precursor of the anti-pernicious anemia factor appears unlikely, in view of the fact that many of the properties of the two materials appear to be quite different, and the best evidence suggests that highly purified anti-pernicious anemia factor does not contain a pterin ring (SubbaRow), and does not yield an aromatic amine, such as *p*-aminobenzoic acid, on hydrolytic cleavage (Stokstad).

The conjugates of folic acid which occur in the diet, although utilized by normal subjects, appear to be inadequately utilized when a deficiency of the anti-pernicious anemia factor exists. The most reasonable hypothesis, based on the data presented, suggests that highly purified liver extracts may supply a substance that is directly or indirectly concerned with the function of at least one of the enzymes which liberate *L. casei* factor from naturally occurring conjugates of the vitamin.

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